Changes in mitochondrial PLIN3 and PLIN5 protein content in rat skeletal muscle following acute contraction and endurance training

Sofhia V. Ramos

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Under the supervision of Sandra Peters, PhD.

Faculty of Applied Health Science
Brock University
St Catharines, Ontario, Canada

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Title: Changes in mitochondrial PLIN3 and PLIN5 protein content in rat skeletal muscle following acute contraction and endurance training
Following Stimulated Contraction

Author: Sofhia V. Ramos

Supervisor: Dr. Sandra J. Peters

Supervisory Committee: Dr. Paul J. LeBlanc

Dr. Wendy E. Ward

External Examiner: Dr. Leslie Stefanyk

ABSTRACT

Surrounding lipid droplets in skeletal muscle are the perilipin (PLIN2-5) family of proteins, regulating lipid droplet metabolism. During exercise lipid droplets provide fatty acids to the mitochondria for oxidation while increasing their proximity to each other. Whether PLIN3 and PLIN5 associate with mitochondria following contraction has not been examined. To determine whether contraction altered mitochondrial PLIN3 and PLIN5 content, sedentary and endurance trained rats underwent acute contraction. The main outcomes are; 1) mitochondrial PLIN3 content is unaltered while mitochondrial PLIN5 content is increased following an acute contraction 2) mitochondrial PLIN3 content is higher in endurance trained rats when compared to sedentary and mitochondrial PLIN5 content is similar in both conditions 3) only PLIN5 mitochondrial content is increased similarly in both groups following acute contraction. This work highlights the dynamics of these two PLIN proteins, which may have roles not only on the lipid droplet but also on the mitochondria.

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List of Abbreviations

ADRP – adipocyte differentiating related protein

ATGL – adipose triglyceride lipase

ATP – adenosine triphosphate

CHO – Chinese hamster ovary

CGI-58 – comparative gene identification- 58

CPT (1 &2) – carnitine palmitoyl transfrase

COX IV – cytochrome c oxidase complex 4

FABPpm – fatty acid binding protein plasma membrane

FABPc – fatty acid binding protein cytosol

FAT/CD36 - fatty acid transport protein/cluster of differentiation 36

GAP – GTPase activating protein

HSL – Hormone sensitive lipase

LSD5P – lipid storage droplet 5 protein

M6PR – mannose-6-phosphate receptor

MGL – monoglyceride lipase

MLDP – myocardial lipid droplet protein

ORO – oil red-O

PAT – perilipin, ADRP, Tip47

PKA – cAMP dependent protein kinase A

PKC – protein kinase C

PPAR – Peroxisome proliferator-activating receptor

SERCA (1&2) – Sarcoendoplamsic reticulum calcium transport ATPase

TIP47 – Tail interacting protein 47

WH – whole homogenate

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CHAPTER 1

Introduction

Excess energy intake into the body results in increased visceral adiposity. Sedentary individuals with excessive visceral adiposity are also known to have high intramuscular lipids (3, 13, 34). This increased intramuscular lipid storage has been shown to interfere with insulin signalling resulting in impaired glucose uptake, which may result in insulin resistance and the development of metabolic disorders such as type 2 diabetes, compromising the quality of life for an individual (27). Interestingly, endurance trained individuals are able to store high amounts of lipids within their skeletal muscle but instead of becoming insulin resistant they become more insulin sensitive (3, 12). This wayward phenomenon is known as the "athletes' paradox", because it was originally believed that the increased intramuscular lipid content was the culprit of the development of insulin resistance (27). Studies now point towards, but are not limited to, toxic lipid intermediates, such as ceramides and diglycerides, as the underlying cause of these metabolic disorders rather than intramuscular triglyceride storage (3, 13).

To better understand the intracellular mechanisms that regulate skeletal muscle metabolism, different methods can be applied that provide a glimpse or snap shot of what may be happening intracellularly. Studying populations in different metabolic states (i.e., normal weight sedentary, overweight, obese, endurance trained) can allow the observation of protein and lipid differences that may contribute to poor health and allow for the development of preventative measures. Research is often conducted in cell culture models in an attempt to control the environment that proteins of interest are exposed to, providing researchers a clear view of what may be happening within the cell. Human and animal models are often used as a more physiological approach where muscle biopsies are volunteered from humans and whole muscle extracted from animals. In addition to

collecting muscle tissue, the individual or animal can be manipulated (i.e., high fat diet, high carbohydrate diet, endurance training, high intensity interval training) to see how certain proteins behave under different metabolic conditions.

This thesis will focus on skeletal muscle, looking at a specific organelle known as the mitochondria. Skeletal muscle mitochondria are responsible for providing the cell with a usable form of energy, adenosine triphosphate (ATP). To do this, a cascade of events occurs to provide a useable fuel, from either sugars (carbohydrates) or fatty acids (triglycerides), which are then transported to the mitochondria to make energy (45, 46). Focusing on lipid metabolism, surrounding lipid droplets in skeletal muscle are members of the perilipin family of proteins (PLIN), which are thought to be involved in regulating the formation and breakdown of lipid droplets (10, 16). Recently, reports have found that PLIN5 also associates with mitochondria in addition to lipid droplets, raising questions of its function in this energy producing organelle. There is a very close association between lipid droplets and mitochondria in skeletal muscle (25, 67, 80), where fatty acids that are released from lipid droplets are directed either to oxidation in mitochondria or to re-esterification. Focusing on PLIN3 and PLIN5, this document will outline the mitochondrial enrichment of these proteins following acute contraction and an 8-week endurance training protocol. The contraction perturbation will be completed *in vivo* by electrically stimulating the sciatic nerve, causing the muscles of the lower limb to contract, inducing lipolysis (75). Primarily subsarcolemmal mitochondria will be isolated for specific protein analysis, in addition to collecting whole muscle for intramuscular lipid and protein analysis. This research will shed some light on how the mitochondrial

content of these PLIN proteins is altered under conditions that would be expected to increase lipolysis and subsequent mitochondrial oxidation of the released fatty acids.

CHAPTER 2

Literature Review

Lipid droplets

In adipose tissue, lipid droplets are thought to be formed between the phospholipid bilayer of the cytoplasmic slide of the endoplasmic reticulum membrane. These lipid droplets bud off coated with a phospholipid monolayer and imbedded proteins that mediate traffic, storage and utilization of neutral lipids within the lipid droplet. Even though there have been extensive studies on the formation of lipid droplets, an exact model has yet to be determined (55, 64, 89, 91). Lipid droplets were initially considered to be storage units, but further studies have revealed a more dynamic function (4). Lipid droplets are believed to move about inside the cell providing substrates to intracellular organelles (95). Lipid droplets contain cholesterol esters, sterol esters and triglycerides, which can be used for the formation of intracellular membranes and as fuel. Triglycerides are the primary storage form of fuel for energy production, and are composed of a glycerol back bone attached to three fatty acids and are utilized for the production of energy in the form of adenosine triphosphate (ATP) in the mitochondria.

Skeletal muscle is able to accept circulating fatty acids in addition to its own endogenous source of fatty acids in the lipid droplets stored under the sarcolemma and between myofibrils (70). Endogenous triglycerides stored in lipid droplets can be hydrolyzed to release fatty acids that can be directly taken to intramuscular mitochondria and used for oxidation which is thought to be a more favourable fuel source during exercise, particularly in endurance trained individuals. Fatty acids released from adipose tissue can also be used as a fuel source during exercise but compared to skeletal muscle endogenous fatty acid storage, adipose tissue fatty acids travel through the circulation bound to albumin, which then requires a host of proteins to mediate its entry into skeletal muscle.

Intramuscular lipid droplets and metabolic disorders

Lipid regulation in skeletal muscle is of particular interest due to the correlation seen with increased lipid content and metabolic disorders such as insulin resistance and type 2 diabetes (13). Interestingly, high lipid content in skeletal muscle is also present in individuals who are endurance trained and highly insulin sensitive (13, 33, 83). This is known as the "athlete's paradox" where both populations have high lipid content within their skeletal muscle but one population is considered to be diseased while the other is healthy. Taking a closer look at the muscle ultrastructure reveals that individuals that are overweight and obese store lipids in a more disorganized fashion where as trained individuals have distinct and larger lipid droplets, placed in close proximity to mitochondria within the fibre where it is believed to be optimal for fuel use (Figure 1) (3). In addition to the fibre lipid content being more organized, individuals who are endurance trained also have more type I and IIa fibres, which are able to store more lipids and primarily rely on fat as a fuel during exercise (83). Further, more recent research has indicated that it is not stored triglycerides per se that are the problem in insulin resistance, but other lipotoxic intermediates that interfere with cell signalling at several levels (3, 4, 12, 13). In obese, sedentary and diabetic populations, training or exercise has been shown to decrease the amount of these potentially toxic lipid intermediates (27, 62, 68, 83). These studies illustrate the physiological importance of exercise training on establishing and maintaining health, and underscore the need to understand how exercise affects the regulation of lipid breakdown. Only a handful of studies have looked at the intracellular proteins that work to mediate skeletal muscle lipolysis and the roles they play with a single bout of muscle contraction or following endurance training (56-58, 65, 66, 71).

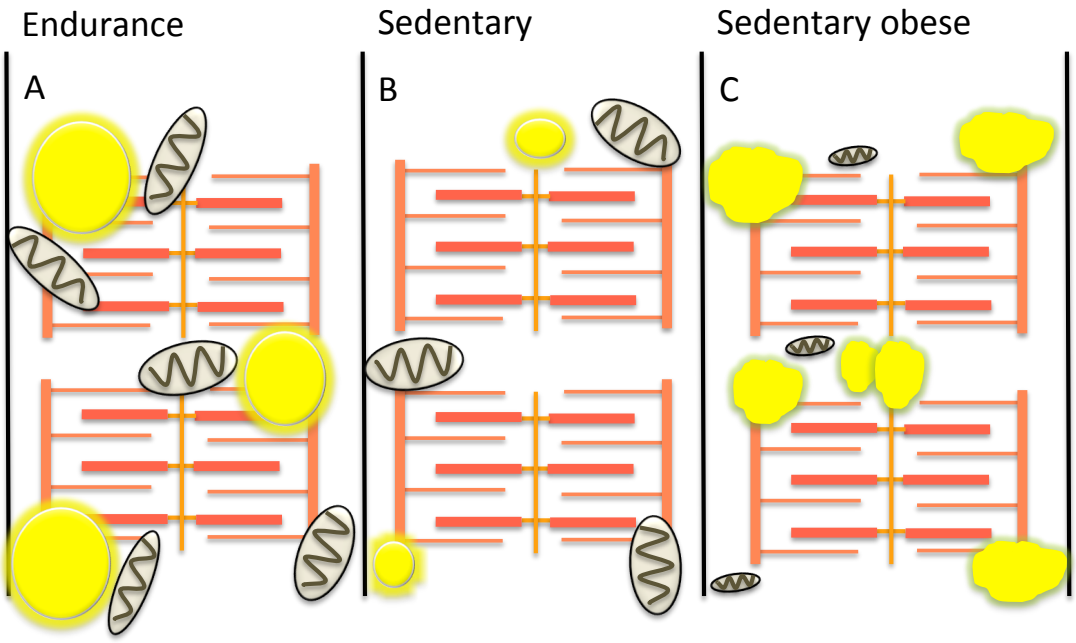

Figure 1. Figure adapted from Amati et al., 2011. (A) Abundant lipid droplet storage in endurance trained individuals' skeletal muscle and is also in close proximity to mitochondria. (B) Moderate amount of lipid storage in skeletal muscle of normal weight sedentary individual with mitochondria still visible. (C) Abundant lipid droplet storage in obese sedentary individual with less intramuscular mitochondrial content than endurance trained and normal weight sedentary individuals.

Skeletal muscle lipolysis

Lipolysis is the sequential process of cleaving fatty acids from triglyceride molecules, which are stored within lipid droplets (2, 18, 70). Lipolysis can be hormonally stimulated by epinephrine and norepinephrine through beta-adrenergic receptors located on the sarcolemmal membrane (54, 87). Activation of this receptor causes the activation of adenylate cyclase, which is responsible for the conversion of an ATP molecule to a cyclic-AMP molecule, a second messenger. This molecule is then able to activate cAMP dependent protein kinase A (PKA), the main kinase responsible for the activation of lipases and proteins involved in lipolysis (54, 87). In addition to beta-adrenergic stimulation activating PKA, skeletal muscle contraction is known to activate extracellular signal-regulated kinase (ERK)/ mitogen activated protein (MAP) kinase 3 through the release of calcium ions (Ca^{2+}), which has also been shown to increase lipolytic rates (26). However, lipolysis can be decreased with the activation of Ca^{2+}dependent calmodulin kinase (CaMK), which is also activated by the release of Ca^{2+}, and AMP-dependent protein kinase, which is activated by elevated intramuscular AMP levels (88). These kinases in turn stimulate the activation or inhibition of the lipases that regulate lipolysis (Figure 2).

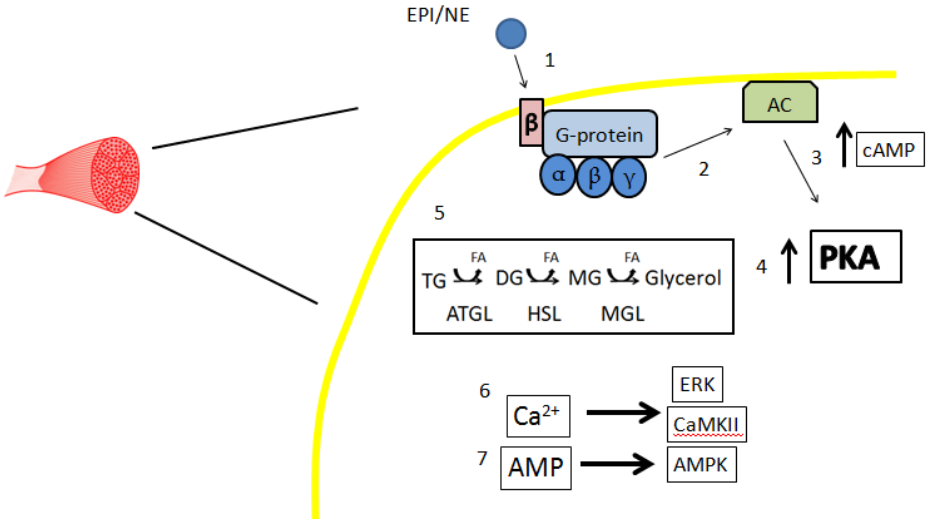

Figure 2. Lipolysis in skeletal muscle. With hormonal stimulation skeletal muscle is able to undergo lipolysis with (1) beta-adrenergic stimulation activating G-proteins (2), which are then able to activate adenylate cyclase converting ATP into cyclic-AMP. (3) Increased cyclic AMP stimulated the activation of (4) cyclic-AMP dependent protein kinase A (PKA), (5) activating the lipases and proteins involved in triglyceride breakdown. In addition to the above mentioned beta-adrenergic pathway, with muscle contraction (6) calcium is released from the sarcoplasmic reticulum and works as a second messenger to activate extracellular related kinase (ERK) and calcium calmodulin kinase II (CaMKII) which can then have an effect on the lipases that regulated lipolysis. In addition to calcium, at higher intensity contractions, intracellular AMP levels raise (7) which can also act as a second messenger activating AMP activated protein kinase (AMPK), which can also alter the rate of lipolysis. (EPI/NE; epinephrine/norepinephrine, AC; adenylate cyclase, TG; triglyceride, DG; diglyceride, MG; monoglyceride, FA; fatty acid, ATGL; adipose triglyceride lipase, HSL; hormone sensitive lipase, MGL; monoglyceride lipase).

The lipases that regulate skeletal muscle lipolysis are adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoglyceride lipase (MGL) (54, 87, 96). ATGL, which is considered to be the rate limiting lipase, initiates lipolysis by cleaving the first fatty acid from a triglyceride molecule when activated by its co-activator comparative gene identification-58 (CGI-58) producing a diglyceride molecule (36, 50, 53, 94). Having a high affinity for diglycerides (31), when activated, HSL moves to lipid droplets to remove a fatty acid from diglyceride molecules leaving behind monoglyceride molecules (37, 66, 84, 86) that can then be hydrolyzed by MGL to produce a fatty acid and glycerol molecule (54) (Figure 2).

The perilipin (PLIN) family of proteins

Lipolysis and the above mentioned lipases are putatively regulated by a family of proteins that surround the lipid droplet membrane called the perilipin (PLIN) family proteins (10, 51). From the discovery of adipose tissue PLIN1 in the laboratory of Constantine Londos, the PLIN family of proteins have been heavily investigated in hopes of developing a better understanding of how lipolysis and lipogenesis is regulated and how these proteins might play a role in metabolic diseases. PLIN1 (perilipin) was the first PLIN family protein to be discovered, and is thought to play a major role in regulating the activation of ATGL and HSL driving lipolysis in adipose tissue (35). PLIN2 (adipose differentiated related protein, ADRP or adipophilin) was the second PLIN family protein to be discovered, and is believed to play a role in lipid droplet formation and fatty acid storage (10, 17). PLIN3 was first discovered as an effector protein for mannose-6-phosphate receptor trafficking and was previously called tail interacting protein of 47 kDa (TIP-47) (1, 6, 10, 20). Later, PLIN3 was found to associate with lipid droplets and

contained a 43% gene sequence homology to PLIN2 (91), thus redefining PLIN3 as a lipid droplet protein. PLIN4, previously named S3-12, was the fourth member to be identified that is heavily expressed in white and brown adipose tissue and is assumed to participate in lipid droplet formation (89). The last PLIN family protein to be discovered was PLIN5, which was previously known as OXPAT (oxidative PAT protein) (also, LD5P and MLDP) (51, 90). This protein was originally named due to its high expression in oxidative tissues (24, 90, 93). The PLIN family members are identified by specific amino acid region called the PAT domain found on PLIN1-3 and PLIN5 in addition to a specific 11-mer repeat region found on all PLIN proteins (Figure 3).

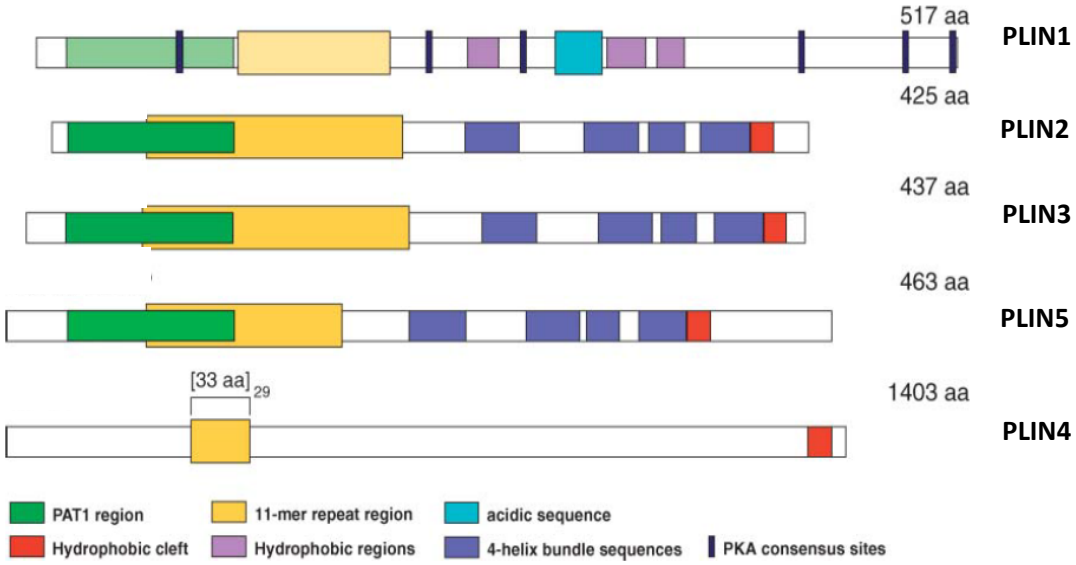

Figure 3. Figure adapted from Brasaemle (2007). Outline of the similarity between the amino acid sequence of the PLIN family of proteins. The green region named the PAT1 region composed of 100 amino acids that are similar on all PLIN proteins except for PLIN4 and is found on the N terminal of the protein. The yellow region (11-mer repeat region) is present on all PLIN family members and consists of amphipathic helices. PLIN1 is the only PLIN family protein with the light blue region (acidic sequence) where that region is polar and 3 hydrophobic regions in purple. PLIN2, PLIN3 and PLIN5 have a similar dark blue region (4-helix bundle sequences) where the protein folds into 4 amphipathic alpha-helices. All of the PLIN family members have a hydrophobic cleft identified as red but PLIN1 and only PLIN1 has known phosphorylation sites identified in black.

Functions for the PLIN family of proteins in adipose tissue are still up for debate, and different investigators have identified many different functions thus far. Studies looking at PLIN protein function tend to be primarily in adipocyte cell culture models because scientists are able to have better control over the environment and stresses the proteins are exposed to (7, 73, 89-91). Many experiments are also conducted in adipose tissue where there is an abundance of lipids, resulting in an optimal location to study this family of lipid droplet proteins (18, 35). Many of these studies use knockdown models where the tissue is genetically manipulated to not express as much of the protein of interest, thus preventing it from having its effect within the cell and potentially pointing at a function for that particular protein (21, 76, 78). Studies also use overexpression; this involves transcriptional modification where more of the particular protein of interest can be expressed amplifying an effect if a particular function (14, 15). Proteomic analysis on amino acid structure has the potential to identify specific regions and sites on the protein, which can identify potential regulation mechanisms of a protein (e.g., phosphorylation sites).

PLIN1

The function of the PLIN family members in skeletal muscle has not been studied to the extent of the research that has been completed in adipose tissue and cell culture models. Skeletal muscle is of particular interest because it is able to store lipid droplets that can be utilized during exercise (25, 57). The PLIN family of proteins possibly regulate the use of triglycerides from lipid droplets during contraction that stimulates lipolysis in skeletal muscle (10, 57-59). Interestingly, PLIN1, the protein with a discrete lipolytic function, is not expressed in skeletal muscle, therefore leaving the regulation of lipogenesis and lipolysis in skeletal muscle relatively unknown. In basal adipose tissue

PLIN1 is found on the lipid droplet membrane bound to CGI-58, where it is believed to be inhibiting lipolysis by sequestering the co-activator for ATGL (9, 19, 35). During lipolysis PLIN1 is phosphorylated on one of its 6 serine residues which causes the release of CGI-58, which can then associate with ATGL, activating it and initiating lipolysis (5, 36). In addition, PLIN1 phosphorylation results in fragmenting of the lipid droplet into smaller droplets, increasing the surface area for lipase access to triglycerides (60). Drawing on what has been done in adipose tissue and cell culture regarding the function of PLIN1-5, inferences can be made in effort to identify discrete roles for these proteins in skeletal muscle.

PLIN2

PLIN2 was the second PLIN family protein identified, with its expression described in various tissues that store lipids (e.g., adipose tissue, liver, heart, skeletal muscle). Immunofluorescence analysis has indicated that PLIN2 is largely found only on the surface of both triglyceride and cholesterol storing lipid droplets, and it is likely targeted for proteasomal degradation when not bound to the lipid droplet (10, 17, 44). This might suggest that PLIN2 is a reliable lipid droplet marker and has been previously used as an intracellular marker of lipid droplets in skeletal muscle (66). Its intracellular location, PAT domain sequence, and regulation by peroxisomal proliferator activated receptors (PPARs) has indicated that PLIN2 is member of the PLIN family of proteins, although a specific function in lipid metabolism has yet to be determined (79). Initially, PLIN2 was suspected to be involved in lipolysis in skeletal muscle due to the absence of PLIN1. With stimulated skeletal muscle contraction PLIN2 localization with lipid droplets was unchanged (57, 66). A human endurance and sprint interval training study examined lipid droplet use, demonstrating that lipid droplets containing PLIN2 were

preferentially used when compared with lipid droplets that did not have PLIN2 (71). These studies reveal that even though there might not be changes with PLIN2 content on lipid droplets, PLIN2 seams to function in lipid droplet breakdown depending on the perturbation applied. A rodent study demonstrated that 30 minutes of *ex vivo* soleus muscle stimulation resulted in protein-protein interactions between PLIN2 and ATGL and, interestingly, not between PLIN2 and CGI-58 (58). This would argue that PLIN2 maybe sequestering the lipase instead of the co-activator in skeletal muscle, inhibiting lipolysis. The PLIN2-ATGL interaction significantly decreased with stimulated contraction and ATGL-CGI-58 interaction increased, suggesting that it is ATGL and not CGI-58 that is sequestered by PLIN2 to prevent high rates of lipolysis under basal conditions in skeletal muscle.

PLIN4

PLIN4 is the fourth PLIN family member identified and the second one thought to be able to exist on and off the lipid droplet, which could suggest a more dynamic role for this protein. PLIN4 is highly expressed in tissues that are specific for lipid storage such as white adipose tissue, and has lower expression in oxidative tissue (10). PLIN4 is the least studied PLIN protein but as its expression is regulated by PPARs, it was suspected to be involved in lipid droplet metabolism. Initially, Wolins and colleagues (92) identified PLIN4 surrounding new forming 3T3-L1 adipocytes treated with a fatty acid medium forming a distinct pool of PLIN4 coated lipid droplets (92). In cardiac muscle of PLIN4 knockout mice, regulation of PLIN5 mRNA expression and protein content was decreased suggesting a teeter-totter regulation of these two PLIN proteins seeing as they have opposite expression in white adipose tissue, liver, cardiac and skeletal muscle (21). In addition, there was reduced cardiac lipid accumulation and no change in protein

content or mRNA expression of PLINs 1-3 showing PLIN4 might play an independent or backup role in lipid droplet metabolism (21). Similarly, with endurance training in obese males and females there was no change in PLIN4 content and therefore its role in lipid metabolism is highly speculative (65).

PLIN3

PLIN3 was the third PLIN family protein discovered due to its 43% sequence homology with PLIN2 (91). Initially, PLIN3 was thought to be involved in intracellular trafficking with its known interaction with the M6PR, mediating vesicle transportation from the Golgi complex to endosomes and back (1, 20); this function will be discussed further below. PLIN3 was believed to be unique because it was thought to be the only PLIN family member whose gene expression was not regulated by PPARs, which are generally activated in situations of increased fat abundance (10). However, some reports analyzing the PLIN3 gene sequence have identified several sites on the PLIN3 protein with the potential to regulate gene expression (PPAR-γ) and protein regulation (cAMP, PKA, and PKC) (81). Supportive physiological evidence for these putative regulators has come from cell culture models where PLIN3 mRNA content is increased with high levels of glucose and insulin (30). Further, *in vivo* treadmill exercise for 1 hour in C57/B16J mice caused immediate increases in PLIN3 mRNA post exercise, with further increases 3 hours post exercise (52). Further work is required to determine how cAMP, PKA, PKC and PPAR-γ effect PLIN3 gene and protein expression.

PLIN3 and intracellular trafficking

The key interaction in the M6PR complex is between PLIN3 acting as an effector protein for a Rab9-GTPase, which is necessary for the complex to mobilize. With the M6PR, PLIN3 is required to allow the binding of the Rab9-GTPase where it is then

hydrolysed by a GTPase-activating protein (GAP), allowing its transportation throughout the cell (1). Understanding how PLIN3 functions with the M6PRs can provide avenues to understanding how PLIN3 might possibly be involved in trafficking lipid droplets or other organelles. PLIN3 is also localized to the endoplasmic reticulum with lipolytic stimulation (e.g., forskolin, a PKA activator) or during lipid synthesis when there is increased cellular oleate uptake and triglyceride formation (73, 91). When analyzing the structure of PLIN3, Hickenbottom and colleagues (38) identified a deep hydrophobic cleft that has the potential to bind small proteins and/or fatty acids which could suggest a potential role for PLIN3 in moving molecules throughout the cell (38).

Being an "exchangeable" lipid droplet protein that may move on and off the lipid droplet, PLIN3 has the potential to play many roles in the regulation of lipid droplet metabolism (lipogenesis and lipolysis) and even the regulation of other intracellular components. Supplementing 3T3-L1 adipocytes with oleate, glucose and insulin allowed for real time visualization of newly forming lipid droplets that were heavily coated with PLIN3 (91). As the lipid droplet matured, PLIN3 content decreased and was subsequently replaced with PLIN2 and then PLIN1. Interestingly, visual observation depicts PLIN3 surrounding smaller lipid droplets that are synthesized away from the larger neutral lipid pool and, as the lipid droplet matures and moves closer to the larger lipid pool, PLIN3 content decreases. Knowing the initial role of PLIN3 was involved with vesicular trafficking, we can speculate that the involvement of PLIN3 with lipid droplet synthesis might be to facilitate the movement of maturing lipid droplets to the larger lipid droplet pool (Figure 4). More recently, Rab40c-GTPase has been implicated in the formation of lipid droplets in Normal Rat Kidney (NRK) epithelial cells with an interaction between

Rab40c and PLIN3 in both 3T3-L1 adipocytes and NRK epithelial cells (77). It is proposed that Rab40c is recruited to the lipid droplets and stimulates the growth of lipid droplets by fusion and fission of smaller lipid droplets (77). Whether PLIN3 is required for this function has yet to be determined, but provides evidence for the proposed role of PLIN3 and lipid droplet movement.

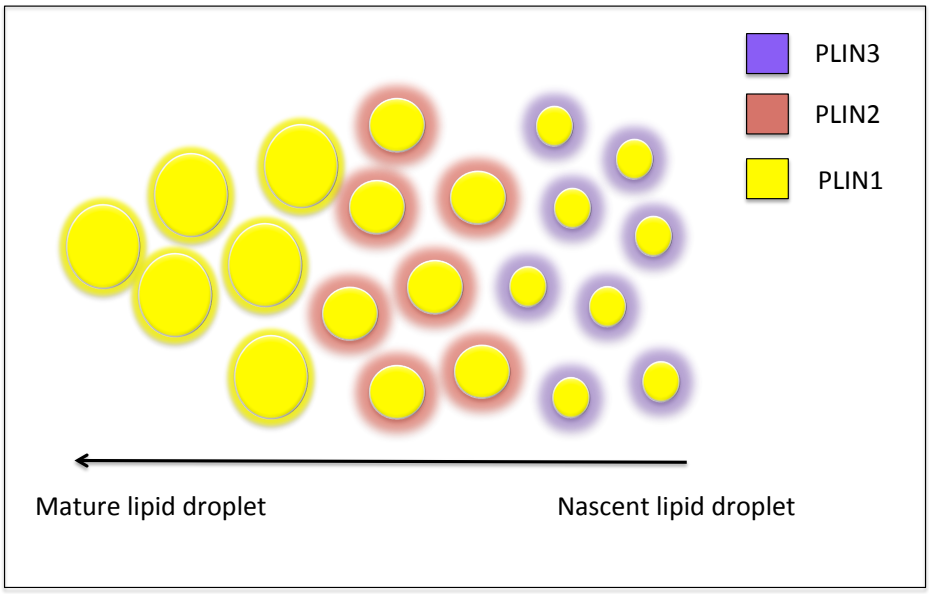

Figure 4. Adapted from Wolins Brasaemle & Bickel (2006). 3T3-L1 adipocytes supplemented with oleate to stimulate lipid droplet synthesis. Newly forming lipid droplets are coated with PLIN3 (purple) and as lipid droplets mature PLIN3 is replaced by PLIN2 (red) and then the mature lipid droplet is coated with PLIN1 (yellow).

Involvement of PLIN3 in lipolysis

A definitive lipolytic role for PLIN3 has yet to be determined in adipose tissue or skeletal muscle. Reports looking at acute lipolytic stimulation to induce lipolysis by epinephrine in primary human myotubes and following a long endurance exercise bout in human vastus lateralis uncovered increased PLIN3 protein content (22). This would suggest that PLIN3 is potentially involved in skeletal muscle lipolysis. In addition, PLIN3 interacts with ATGL, CGI-58 and HSL, all major players in the regulation of lipolysis in skeletal muscle (58, 59). To functionally understand these interactions, isolated soleus muscles were stimulated to contract for 30 minutes at an intensity known to stimulate muscle lipolysis (58, 59). This resulted in a decreased protein-protein interaction between PLIN3-HSL, PLIN3-ATGL and no change in PLIN3-CGI-58. Coupling these interactions with the increased protein content following acute lipolytic stimulation suggest that PLIN3 might play a functional role in the breakdown of fatty acids.

PLIN3 interaction with other cellular organelles and proteins

The diversity of potential roles for PLIN3 continues to expand when looking outside the 'lipid research' or generalized vesicle trafficking. In human cervical cancer HeLa cells exposed to oxidative stress, PLIN3 was shown to localize to the mitochondria, a major target of oxidative stress, to prevent cell death (39). Mitochondrial membrane potential was measured in these cells and with the presence of PLIN3; the membrane potential was maintained and thought to be responsible for the prevention of cell death. This mitochondrial-PLIN3 interaction was also identified in isolated rat hepatic tissue proposing the same mechanisms (40). In addition, as described above, PLIN3 also interacts with the endoplasmic reticulum in cell culture models where it is thought to play

a role in the formation of lipid droplets and recruited away from the endoplasmic reticulum with lipolytic stimulation (73). Together, PLIN3 interaction with cellular organelles and receptor proteins displays the ability of PLIN3 to localize throughout the cell when stimulated, potentially to carry out a specific function(s).

PLIN3 summary

As described above PLIN3 seems to be involved in both spectrums of lipid droplet metabolism, from lipid droplet formation to breakdown. Cell culture models are in support of a role for PLIN3 in lipid droplet formation with a higher PLIN3 lipid droplet protein content on nascent lipid droplets that decrease as the lipid droplet matures and moves to the larger lipid droplet pool. In addition, PLIN3 may also function with intracellular trafficking as it was first discovered with M6PR trafficking. Being an exchangeable lipid droplet protein PLIN3 is able to exist on the lipid droplet and in the cytosolic environment with the ability to move to different intracellular organelle depending on the stress presented to the cell. In skeletal muscle PLIN3 is thought to be involved in lipolysis as it interacts with the main lipases (ATGL and HSL) and co-lipase (CGI-58) at rest and following stimulated contraction. What remains unknown is whether PLIN3 interacts with other organelle in skeletal muscle and whether this is altered with stimulated lipolysis.

PLIN5

PLIN5 was the last PLIN family member to be discovered and has an amino acid sequence that is 30% and 25% identical to PLIN3 and PLIN2 respectively (90). Like PLIN3 and PLIN4, PLIN5 is an exchangeable lipid droplet protein, meaning it can be found on the lipid droplet membrane and in the cytosolic environment. Like the other PLIN family proteins, expression of PLIN5 is regulated by PPARs and it is highly

expressed in brown adipose tissue, heart, skeletal muscle and liver with a lower expression in white adipose tissue (10, 11, 90). The differential tissue expression of PLIN5 would suggest that this protein is more involved in lipid breakdown because its expression is highest in more oxidative tissues that use triglycerides for energy production (90).

However, PLIN5 has been implicated in both ends of the lipid metabolism spectrum; where it has also been shown to assist in triglyceride accumulation in a variety of animal and human liver, skeletal muscle and cell culture models (11, 63, 90), and also assists with break down of lipid droplets with adrenergic stimulation (15, 58, 72). To support a function in triglyceride accumulation, COS7 kidney cells with ectopic expression of PLIN5 resulted in a significant triglyceride accumulation when compared to their controls (90). A study done with Chinese hamster ovary (CHO) cells speculated that cytosolic PLIN5 is involved with lipid accumulation by measuring PLIN5 on smaller cytosolic lipid droplets, where they believe PLIN5 is involved in retrieving smaller lipid bodies in the cytosol and localizing them to the larger lipid droplet pool (7). This study suggests a novel trafficking role for PLIN5, which has not been adequately investigated.

PLIN5 involvement in lipolysis

As described earlier, PLIN5 has been implicated in to be involved in lipolysis. Work in our laboratory studied PLIN5 in skeletal muscle in response to acute *ex vivo* contraction, and supports the involvement of PLIN5 in lipase regulation (58, 59). Results from these studies identified PLIN5 protein-protein interactions with ATGL, CGI-58 and HSL under resting, contracted, epinephrine and the combination of epinephrine and contraction states (58, 59). The PLIN5-ATGL and PLIN5-CGI-58 interaction was also

measured without change in human vastus lateralis following 60 min of moderate intensity exercise (61). These protein-protein interactions between PLIN5 and lipases and co-lipases during rest and contraction highlight the complexity of skeletal muscle lipolysis regulation requiring further research to fully understand how lipases are regulated. In addition to the protein-protein interactions of PLIN5, under fasted conditions in livers of C57BL/6 mice, PLIN5 protein content significantly increased when compared to fed mice (90). This would indicate that under situations such as fasting where the body is more reliant on fat as a fuel, PLIN5 protein is increased, potentially aiding in lipid droplet breakdown. Although there is no net change in co-localization of PLIN5 to the lipid droplet in isolated contracting muscle or in exercising human muscle (57), it is still not known whether there is PLIN5 association with other organelles during muscle contraction.

PLIN5 and the mitochondria

The benefits of endurance training on skeletal muscle are not limited to, but include increased lipid droplet storage and increased oxidative capacity, which can be measured by increased mitochondrial content. As mentioned above, endurance trained individuals are insulin sensitive regardless of the high intramuscular lipid content. Therefore, there may be a communicating link between lipid droplets and mitochondria that may be important in preserving skeletal muscle health when intramuscular lipids are increased. PLIN5 expression is closely mirrored to a tissue's oxidative capacity (23, 65, 90), which then suggests that PLIN5 may be involved in the communication between lipid droplets and mitochondria.

In 2012, Bosma and colleagues (14) identified PLIN5 in skeletal muscle mitochondria from rat tibalis anterior muscle, while Wang and colleagues (85) also identified PLIN5 in cardiac muscle mitochondria in Wistar rats. However, whole muscle PLIN5 overexpression resulted in no change in the proteins involved in oxidative phosphorylation and did not alter fatty acid oxidation in mitochondria extracted from overexpressing muscle (14, 15). These results would suggest that PLIN5 might not have a functional role in mitochondria, as overexpression did not affect the mitochondrial machinery or its function. However, when PLIN5 was overexpressed in whole muscle the rate of ^{14}C-palmitate oxidation was significantly increased even in the face of unaltered mitochondrial oxidative capacity, potentially supporting a role for PLIN5 in facilitating or regulating fatty acid transfer to the mitochondria (14, 15).

More recently, Mason and colleagues (61) examined the intracellular location of PLIN5 in skeletal muscle in addition to analyzing its co-localization with ATGL and CGI-58 at rest and following 60 min of moderate intensity exercise. Here they report no change in PLIN5-mitochondrial co-localization following exercise (61). Interestingly, this model did not result in a change in intramuscular lipid content measured by oil red-O, which might suggest that the exercise intensity was not strong enough to elicit a strong lipolytic stimulus. What remains unknown is whether the association between PLIN5 and mitochondria changes under conditions of altered intramuscular lipid content.

PLIN3 and PLIN5 protein response to endurance training

To our knowledge, only two other laboratories have studied the PLIN family of proteins in skeletal muscle following endurance training, focusing on total muscle content of the PLIN proteins. Because the PLIN family of proteins have been implicated in the

regulation of lipid droplet breakdown and endurance trained individuals are thought to rely more heavily on intramuscular triglycerides during prolonged exercise it is essential to know how the PLIN family of proteins respond under these stresses. The first study to do this looked at PLIN family proteins in response to a 12-week endurance training program in lean and obese individuals and whether PLIN protein content was related to intramuscular lipid content and/or mitochondrial content (65). The second study aimed to identify the effects of endurance training and lipolytic proteins in obese males following an 8-week program (56). Lastly, the third study analyzed the localization and content of PLIN2 and PLIN5 following sprint interval and endurance training in sedentary males (72). Focusing on results for PLIN3 and PLIN5 with endurance training we see an increase in both PLIN3 and PLIN5 in whole muscle. Interestingly, Shepherd and colleagues (72) found that lipid droplets coated with PLIN5 were preferentially used over lipid droplets not coated with PLIN5 after both sprint interval and endurance training. They also reported a decreased lipid droplet density and also a decrease in PLIN5 following their post-training exercise test. What was not measured was whether this decreased PLIN5 content on lipid droplets was mirrored by PLIN5 enrichment in another intracellular organelle.

Intramuscular lipid metabolism

Intramuscular triglyceride use during exercise and muscle contraction

Glycogen and fatty acids are the primary fuel sources for the body and are both mobilized during exercise. Particularly, during prolonged moderate intensity exercise, the body is thought to rely more heavily on fatty acids as a fuel. There are opposing results on whether endurance training increases the reliance on intramuscular triglycerides or not. Intramuscular lipid use in endurance-trained individuals has been shown to positively

correlate with insulin sensitivity, which was thought to be the underlying distinction in the athletes' paradox (13, 69). Unfortunately, studies have reported variable results with some trained subjects relying more on intramuscular lipids while others not (Table 1 and 2). The most variation can be seen in human models using muscle biopsies from the vastus lateralis. Here these studies are able to report an increase in intramuscular lipid utilization following endurance training using pre and post measures of lipid content, but many do not reach statistical significance (8, 28, 49). This could be due to measurement variability associated with extracting the muscle, regional difference biopsy sampling relative to lipid deposition, the population characteristics (obese, lean, trained, normal weight) and also the method used to measure the amount of intramuscular triglycerides (e.g., biochemical, mass resonance spectrometry, immunofluorescence microscopy) (29, 82).

Table 1. Summary of reports that have measured intramuscular lipid use following endurance training in animal models

Author	Animal/Muscle	Training	IMTG Reliance
Mole et al., 1971	Male Wistar Rats/ Red gastrocnemius and quadriceps	Running: 12wks, 5d/wk, 2h/d. 8 incline, 31m/min (12 1min intervals @ 42m/min	↑ Reliance
Davies et al., 1980	Female Wistar Rats/ Mixed hindlimb	Running: 10wks, 5d/wk, 2h/d. 15% incline, 26.8m/min	↑ Reliance
Dyck et al., 2000	Female Sprague Dawely Rats/ Soleus	Running: 8wks, 5d/wk, 2h/d. 15% incline, 31m/min	↓ Reliance

Table 2. Summary of studies analyzing intramuscular lipid use following endurance training in human models

Author	Population	Training	IMTG Reliance
Hurley et al., 1986	Young Males	Cycle, 12wks, 3d/wk = sprints, 3d/wk = 40 min at 75% VO_2max	↑ Reliance
Kines et al., 1993	Healthy Males	Dynamic knee extension, 8wks, 4d/wk, 2h	— Reliance
Phillips et al., 1996	Healthy untrained Males	Cycling, 31d, 3h/d at ~60% VO_{2peak}	↑ Reliance
Bergman et al., 1999	Untrained Young Males	Cycle, 9wks, 5d/wk, 75% VO_2max for 1h	— Reliance
Tarnopolsky et al., 2007	Young Females and Males	Cycling, 7wks, 4-5d/wk, 1h at ~60% VO_{2peak}	↑ Reliance
Dube et al., 2008	Sedentary Females and Males	Walking/Cycling, 16wks, 4-5d/wk, 45min	↑ Reliance

Free fatty acid entry into skeletal muscle

Fatty acids entering skeletal muscle from the circulation are mediated by specialized proteins; fatty acid binding protein plasma membrane (FABPpm), fatty acid bind protein cytosol (FABPc), fatty acid translocase/cluster of differentiation 36 (FAT/CD36) and fatty acid transport proteins (FATP/solute carrier family 27) (32, 47, 48). The exact mechanism and regulation of these proteins remain unknown but they are known to mediate the influx of albumin bound fatty acids from circulation into the cell and either to lipid droplets or the mitochondria where another set of regulating protein allow the flow of fatty acids into the mitochondria for beta-oxidation. As described above, it is believed that toxic lipid intermediates inhibit the insulin signalling pathway by activating protein kinase C (PKC), which prevents the activation of Akt in the insulin signalling pathway therefore disrupting skeletal muscle glucose uptake (13, 43). Understanding the dynamics of fatty acid transportation, esterification, and use in skeletal muscle could provide the missing link to understanding the development of insulin resistance and other musculoskeletal disorders.

Fatty acid entry into mitochondria

During energy demands, fatty acids that enter skeletal muscle, or hydrolysed from a triglyceride molecule in lipid droplets within skeletal muscle, are shuttled to the mitochondria where they will be utilized for the synthesis of ATP. The transport of fatty acids across the double bilayer membrane of the mitochondria is a highly regulated (47). Briefly, fatty acids are converted into a fatty acyl-CoA molecule, which is then subsequently converted into an acyl-carnitine by the rate limiting enzyme carnitine palmitoyl transferase 1 (CPT1) to allow it through the outer membrane. Once through the outer membrane the acyl-carnitine is transported in-between the inner and outer

membrane by translocase. Once at the inner membrane, the acyl-carnitine is transported through and converted back into an acyl-CoA molecule by carnitine palmitoyl transferase 2 (CPT2) (43, 46, 47). When fatty acids are hydrolysed from the lipid droplet in skeletal muscle they may be re-esterified or shuttled to the mitochondria for oxidation (74). How this fatty acid selection process process occurs is relatively unknown. PLIN3 and PLIN5 show promise as potential mediators of fatty acid or lipid droplet interaction with skeletal muscle mitochondria (1, 7) as they are both exchangeable lipid droplet proteins that interact with intracellular organelle. This is tempting to speculate since PLIN3 has a known role with vesicular transportation in cell culture models (1, 20) and PLIN5 localizes to mitochondria in skeletal muscle models. However, the nature of their association with the mitochondria and what might occur under lipolytic conditions is unknown.

Lipid droplet-mitochondria interaction

The mitochondrial reticulum in skeletal muscle has a strong locational relationship with lipid droplets in skeletal muscle (67, 70). Lipid droplet formation seems to be particularly placed in areas within the muscle fibre where it would be efficient for fuel provision to the mitochondria (67). Developing a better understanding of how the lipid droplet-mitochondria interaction works can play a major role in uncovering the underlying mechanisms behind insulin resistance and type 2 diabetes in overweight and obese populations. Endurance training has been shown to increase oxidative capacity of skeletal muscle, with type I fibres able to store more lipids and generate more proteins involved in fat oxidation such as citrate synthase, succinate dehydrogenase, and cytochrome c (41, 42, 80). In addition, training results in increased number of mitochondria “touching” intramuscular lipid droplets as visualized by transmission

electron micrographs (80) (Figure1). This increased lipid droplet-to-mitochondrion interaction was also seen after a single bout of moderate intensity exercise in women, indicating how dynamic these two organelles can be (25, 68). Lipid droplet composition and distribution throughout skeletal muscle is becoming an important part of the development of insulin resistance and it is clear that not only lipid droplet regulation by the PLIN family proteins is important, but also the study of these two organelles together should be a field of great importance, but has not yet been investigated.

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CHAPTER 3

Statement of the problem/purpose

The main aim of this thesis is to characterize skeletal muscle mitochondrial PLIN3 and PLIN5 content prior to and following acute electrically stimulated muscle contraction that is known to induce muscle lipolysis. A secondary purpose was to see if this would be altered following an 8-week endurance training study that might be expected to increase reliance on intramuscular triglycerides for energy provision. The lipolytic function of these two PLIN proteins in skeletal muscle is currently unknown and whether their function involves the mitochondria is currently unknown. This was completed in two studies outlined in Chapter 4 (Study 1: Higher PLIN5 but not PLIN3 content in isolated skeletal muscle mitochondria following acute *in vivo* lipolytic contraction in rat hindlimb) and Chapter 5 (Study 2: Changes in rat skeletal muscle mitochondrial PLIN3 and PLIN5 protein content following 8-weeks of endurance training and acute electrically stimulated contraction).

The main purpose of Study 1 (Chapter 4) was to a) determine PLIN3 protein is present in skeletal muscle mitochondria; and b) to determine any change in PLIN3 or PLIN5 protein content in skeletal muscle mitochondria following a 30 min *in vivo* electrically stimulated contraction period to elicit lipolysis. A secondary purpose of this study was to determine if an interaction exist between PLIN3 and PLIN5 and whether this interaction changes following contraction. The second study completed (Chapter 5) aimed to determine if PLIN3 and PLIN5 protein is increased in the mitochondrial fraction of skeletal muscle following an 8-week treadmill running protocol in rats. In addition, we set out to determine whether there would be a change in the degree of PLIN3 and PLIN5 mitochondrial enrichment following 30 min of *in vivo* electrically stimulated muscle

contraction designed to elicit lipolysis when comparing sedentary and endurance trained rats.

Chapter 4

Study 1

Higher PLIN5 but not PLIN3 content in isolated skeletal muscle mitochondria following acute *in vivo* lipolytic contraction in rat hindlimb

Sofhia V. Ramos[1,3], Rebecca E. K. MacPherson[3], Patrick C. Turnbull[1,3], Kirsten N. Bott[1,3], Paul LeBlanc[2,3], Wendy E. Ward[1,2,3], Sandra J. Peters[1,3]

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Affiliations

[1]Department of Kinesiology, [2]Department of Health Science, [3]Center for Bone and Muscle Health, Brock University, 500 Glenridge Ave, St Catharines, ON L2S 3A1

Contributions:

Sofhia Ramos- Conceived and designed the study, developed the model, performed the experiments, did the biochemical analysis, analyzed and plotted data, wrote manuscript, submitted manuscript.
Rebecca MacPherson- Assisted in design/conception of the study, assisted in developing the model, edited manuscript drafts, approved final manuscript.
Patrick Turnbull- Assisted with the design of the study, provided intellectual input, edited manuscript drafts, approved final manuscript.
Kirsten Bott- Performed experiments related to PLIN3: PLIN5 association, edited manuscript drafts, approved final manuscript.
Paul LeBlanc- Assisted with the design of the study, assisted with biochemical analysis, edited manuscript drafts, approved final manuscript.
Wendy Ward- Assisted with the design of the study, provided intellectual input, edited manuscript drafts, approved final manuscript.
Sandra Peters – Academic supervisor, helped to conceive and design the study, edited manuscript drafts, approved final manuscript, corresponding author.

Abstract

Contraction mediated lipolysis increases the association of lipid droplets and mitochondria, indicating an important role in the passage of fatty acids from lipid droplets to mitochondria in skeletal muscle. PLIN3 and PLIN5 are of particular interest to the lipid droplet-mitochondria interaction because PLIN3 is able to move about within cells and PLIN5 associates with skeletal muscle mitochondria. This study primarily investigated 1) if PLIN3 is detected in skeletal muscle mitochondrial fraction and 2) if mitochondrial protein content of PLIN3 and/or PLIN5 changes following stimulated contraction. A secondary aim was to determine if PLIN3 and PLIN5 associate and whether this changes following contraction. Male Long Evans rats (n= 21; age, 52 days; weight= 317 ± 6g) underwent 30min of hindlimb stimulation (10ms impulses, 100Hz/3s at 10-20V; train duration 100ms). Contraction induced a ~50% reduction in intramuscular lipid content measured by oil red-O staining of red gastrocnemius muscle. Mitochondria were isolated from red gastrocnemius muscle by differential centrifugation, and proteins were detected by Western blotting. Mitochondrial PLIN5 content was ~1.6-fold higher following 30min of contraction and PLIN3 content was detected in the mitochondrial fraction, but unchanged following contraction. An association between PLIN3 and PLIN5 was observed and remained unaltered following contraction. PLIN5 may play a role in mitochondria during lipolytic contraction, which is consistent with a role in facilitating/regulating mitochondrial fatty acid oxidation. PLIN3 and PLIN5 may be working together on the lipid droplet and mitochondria during contraction induced lipolysis.

Key Words: OXPAT, TIP-47, perilipin family, intramuscular triglycerides, lipolysis

Purpose

The regulation of lipid droplet metabolism in skeletal muscle is relatively unknown although, it is believed that the PLIN family of proteins are involved. In 2010, two laboratories made the discovery of PLIN5 being associated with skeletal muscle mitochondria but its function remains unknown in addition to whether any other PLIN members are associated with the mitochondria. The purpose of this chapter is to identify whether PLIN3 associates with skeletal muscle mitochondria. We also aim to determine whether PLIN3 or PLIN5 mitochondrial protein content changes following acute contraction that induces lipolysis. As PLIN3 and PLIN5 have been shown to interact with all skeletal muscle lipases and co-activators set out to determine if PLIN3 and PLIN5 have a protein-protein interaction and whether this changes following acute contraction.

Hypothesis

1. Isolated subsarcolemmal mitochondria from red gastrocnemius muscle will contain PLIN3 protein in resting muscle.
2. Following electrically stimulated *in vivo* lipolytic contraction, mitochondria isolated from red gastrocnemius muscle will have an increased PLIN3 and PLIN5 protein.
3. At rest and following *in vivo* lipolytic contraction, PLIN3 and PLIN5 will be associated and remain unchanged in whole homogenates from red gastrocnemius muscles.

Introduction

Lipid droplets are dynamic energy storage organelles, which function to store neutral lipids and are found in most tissue types (e.g., adipose tissue, skeletal muscle and bone) (5, 11, 46). Coated with a phospholipid monolayer, lipid droplets house triglycerides, sterol esters and ceramides that can be used as substrates for the formation of intracellular membranes or energy production in the form of ATP (38, 46, 50, 66). Bound to and embedded in the lipid droplet membrane are the perilipin (PLIN1-5) family of proteins, which are believed to be involved in lipid droplet formation and the breakdown of stored lipids (26, 39, 52). Of the PLIN family, PLIN3 and PLIN5 are found on the lipid droplet and in the cytosolic environment, interacting with other intracellular organelles and proteins (4, 42, 43, 56, 63). The role of PLIN3 and PLIN5 in the liberation of fatty acids directly from lipid droplets is currently unknown. Further, it is unknown if interactions of the PLINs with other intracellular organelles, such as mitochondria, are involved in lipid droplet breakdown and/or subsequent oxidation of the released fatty acids. In adipose tissue, fatty acids released from lipid droplets are sent into circulation, while in skeletal muscle, fatty acids are either taken up by mitochondria for oxidation or re-esterified in the cytosol (37, 50). To this end, muscle lipid droplets are known to interact with mitochondria (51, 54), but how this interaction occurs is poorly understood.

Endurance training in both male and female human subjects resulted in a decreased distance between lipid droplets and mitochondria, suggesting that the lipid droplet and mitochondria physically moved closer to each other. This is consistent with the increased reliance on intramuscular triglycerides as a fuel, typically seen post-training (33, 45, 60). The association between lipid droplets and mitochondria is also observed following an acute bout of exercise in female participants who are known to rely heavily

on fat oxidation after an acute bout of exercise (21). Thus, it appears that the lipid droplet and mitochondria in skeletal muscle work together or interact to promote efficient supply of fatty acids during acute and chronic exercise. However, the mechanism(s) governing this interaction and whether the proteins associated with the lipid droplet or mitochondria are involved is currently unknown.

PLIN5 is thought to play a role in fatty acid oxidation as it is predominantly expressed in tissue with high oxidative capacity, such as skeletal muscle. Whole muscle overexpression of PLIN5 up-regulates the expression of oxidative enzymes in skeletal muscle (13, 62). More recently, PLIN5 protein content was detected in mitochondria from skeletal muscle and cardiac tissue (12, 64). Mitochondria isolated from PLIN5 overexpressing rat tibalis anterior muscle did not augment mitochondrial density or respiration but increased ^{14}C-palmitate oxidation in overexpressing whole muscle suggesting that its function might not be involved in mitochondrial mechanics but rather the fate of fatty acids when hydrolyzed (12, 61). With the novel finding of a lipid droplet associated protein, PLIN5, found within skeletal muscle mitochondria, it is of special interest because with endurance training there is an increased lipid content, mitochondrial content and PLIN5 content (40, 49, 55). Therefore, it is thought that PLIN5 may be a communicating link between the lipid droplet and mitochondria (12, 36).

It is currently unknown if any other PLIN family proteins interact with skeletal muscle mitochondria, however PLIN3 may be a likely candidate. PLIN3 was initially discovered by its involvement with the mannose-6-phosphate receptor, as an effector protein for Rab9 to the receptor complex to initiate intracellular vesicular movement (2, 22). Interestingly, in HeLa cells placed under oxidative stress PLIN3 was recruited to the

mitochondria and was able to stabilize the mitochondrial membrane preventing apoptosis (29). While this intracellular function is still under investigation (2, 7, 15, 22), this study exemplifies the ability of PLIN3 to move from the cytosol to the mitochondria under a specific perturbation such as the introduction of oxidative stress. What remains unknown is whether PLIN3 can be recruited to the mitochondria under lipolytic conditions, as a function in skeletal muscle for this protein is currently unknown.

Previous work has identified interactions using co-immuoprecipitation methods, between PLIN3 and PLIN5 with ATGL, HSL and CGI-58 at rest, with no change in this interaction following contraction (42, 43). Moreover, PLIN3 and PLIN5 have been shown to inhabit the same smaller cytosolic lipid droplets in Chinese Hamster Ovary cells, potentially bringing those smaller lipid droplets to the larger lipid droplet pool or trafficking them for oxidation (8). During rest, lipolytic contraction, epinephrine or the combination of lipolytic contraction and epinephrine, PLIN3 and PLIN5 appear to be serine phosphorylated, therefore leaving the mechanism(s) of regulation unknown. As PLIN3 and PLIN5 interact with similar lipolytic proteins, potentially inhabit the same smaller lipid droplets and are serine phosphorylated under the above-mentioned conditions, would suggest that PLIN3 and PLIN5 might interact. Whether this interaction exists and changes with lipolytic contraction is currently unknown.

Research thus far points towards a potential role for PLIN3 and PLIN5 in contraction-mediated lipolysis in skeletal muscle, particularly with respect to a role in the mitochondria. The main purpose of this study is a) to determine if PLIN3 protein is present in skeletal muscle mitochondria; and b) to determine any change in PLIN3 or PLIN5 protein content in skeletal muscle mitochondria following a 30 min *in vivo*

electrically stimulated contraction period to elicit lipolysis (58). We hypothesize that PLIN3 will be present in rested skeletal muscle mitochondria and that the mitochondrial protein content of both PLIN3 and PLIN5 will increase with stimulated contraction. A secondary purpose of this study is to determine if an interaction exists between PLIN3 and PLIN5 and whether this interaction changes following contraction. We hypothesize that this interaction will not change as seen with the other reported interactions between these PLIN proteins and skeletal muscle lipases and co-activators (42, 43).

Methods

Animals

Male Long-Evans rats (n= 21, body weight= 317±6g) at approximately 3 months of age were used for this study. Rats were purchased from Charles River Laboratories (Canada). All procedures and protocols were approved by the Animal Care and Utilization Committee at Brock University and conform to all Canadian Council on Animal Care guidelines (47). Rats were housed in pairs in the Comparative Bioscience Facility, maintained on a 12:12 light-dark cycle, fed standard rodent chow, Tekland Global 14% protein, (Harlan Tekland Global, Mississauga, ON, Canada) and had ad libitum access to food and water.

Sciatic nerve stimulation protocol

Anaesthetized rats underwent sciatic nerve stimulation for 30 min (10ms impulses, 100Hz/3s at 10-20V; train duration 100ms), which has previously been shown to induce lipolysis in rat hindlimb muscles (58). This protocol consists of two 13 min halves with a 4 min break in between. Rats were anaesthetized with sodium pentobarbital (6mg/100g of body weight) via intraperitoneal injection. A small incision was made on

the left leg above the hip to expose the sciatic nerve. Curved platinum electrical wires were attached to the sciatic nerve for stimulation with the left leg being stimulated while the right remained as a resting control. Upon completion of the stimulated contraction, the plantaris muscle (n= 5) was removed and prepared for mechanical sarcolemma isolation (25). Red gastrocnemius muscles were then excised and divided as follows; the belly of the muscle was cut and set in embedding compound (Cryomatrix, Pittsburgh, PA) and cooled in methyl-butane for 90 seconds before being stored at -80°C. From the remaining red gastrocnemius, approximately 30mg was cut off (n= 9) and snap frozen for protein analysis, while the remaining muscle (n= 15) was prepared for primarily subsarcolemmal mitochondria isolation.

Skeletal muscle lipid staining

Embedded red gastrocnemius muscles (n= 11) were cut into transverse sections using a cryotome (ThermoShandon, Runcorn, Cheshire, UK) set at -20°C, cutting 10µm thick sections, and then mounted on microscope slides. Microscope slides containing muscle sections were fixed in 3.7% formaldehyde for 1hour followed by a 30 min immersion in a diluted working solution of oil red-O (oil red-O; O0625; Sigma-Aldrich, St. Louis, MO) as described in Koopman et al., (35) and previously done in our lab (41-43). Slides were then washed 3 times, 5 min per wash, with deionised water. Once dried, each muscle section was coated with 10µl of anti-fade reagent (no. P36930; Prolong Gold Anti-fade Reagent; Invitrogen) and covered with a glass cover slip. Slides were stored in the dark overnight to dry.

Imaging and analysis

Muscle sections were analyzed using a Nikon Eclipse 80i fluorescence microscope (Nikon Eclipse 80i; Chiyoda-ku, Tokyo, Japan) and images were captured with a digital camera attached to the microscope (Retiga 1300, QImaging, Burnaby, BC, Canada). Oil red-O stain was visualized with 550 fluorophore. Images were captured at 40X magnification, with three fields of view/muscle cross section (18.8 ± 0.98 fibres/field of view). Each fibre (57 ± 3.00 fibres/sample) was manually outlined to determine lipid content within each fibre using imaging software (NIS-Elements AR 3.00; Nikon Instruments, Melville, NY). Lipid content was quantified by manually selecting an intensity threshold that was applied to all images. The number, area and objects within the fibres emitting a fluorescent signal were recorded and expressed as the fraction of the measured area that was stained.

Subsarcolemmal mitochondrial isolation and purification

The mitochondria isolation protocol described has been adapted from previous methods and used in our lab (34, 48, 59). Briefly, fresh muscles were directly placed on an inverted glass plate, on ice and manually minced. Samples were then immersed in 20 times the volume of solution 1 (100mM KCl, 40mM Tris HCl, 10mM Tris base, 5mM $MgSO_4$, 5mM EDTA and 1mM ATP) and manual homogenized with a glass homogenizer. Samples underwent differential centrifugation; homogenate was centrifuged for 10 min at 700g, and supernatant was collected and spun at 14000g to extract mitochondria. Mitochondria were then re-suspended in 10 times the volume of solution 2 (100mM KCl, 40mM Tris HCl, 5mM Tris base, 1mM MgSO4, 0.01mM EDTA, 1% BSA and 0.25mM ATP) and washed for 10 min at 7000g followed by a wash in solution 3 (100mM KCl, 40mM Tris HCl, 5mM Tris base, 1mM MgSO4, 0.01mM

EDTA, and 0.25mM ATP) for 10 min at 7000g. Mitochondria were further purified with a 60% Percoll ® (P1644, Saint Louis, MO) gradient and re-suspended in sucrose and mannitol solution (220mM sucrose, 70mM mannitol, 10mM Tris HCl and 0.1mM EDTA). Samples were stored at -80°C until protein analysis.

The purity of the mitochondrial fraction was determined by Western blotting. Contamination for sarco (endo) plasmic reticulum was measured by SERCA1 and SERCA 2 protein content, lipid droplet contamination was assessed by PLIN2 protein content, and sarcolemma contamination was measured by beta-dystroglycan protein content. All membranes were cut at ~25kDa band and probed for COX IV (10 kDa) to confirm the presence of mitochondria and used as a loading control.

Mechanical sarcolemma isolation

As a precaution, sarcolemmal cuffs were collected from the plantaris muscle to determine if PLIN3 and/or PLIN5 content were present in the sarcolemma and/or if it changed with contraction as seen with PLIN1B and insulin stimulation in human adipocytes (1). Sarcolemmal cuffs were isolated as previously described (25). Briefly, fibres were isolated using microdissecting forceps and visualized with a dissecting microscope (Nikon SMZ645 with a Nikon 3002752 objective and Nikon C-W10 9 A/22 eyepiece). Plantaris muscles were placed in a petri dish at room temperature suspended in Sylgard with a resting solution (Sylgard 184, DOW Corning) (90mM 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 50mM ethylene glycol tetraacetic acid, 10.3 mM magnesium oxide, 8 mM ATP, 10mM creatine phosphate, pH 7.1 with 4 M sodium hydroxide). A group of fibres were pulled away from the muscle belly, teasing out

individual fibres. Individual fibres were then grabbed and pulled at each end, splitting it apart until the sarcolemma rolled back on its self, forming a cuff. For each cuff collected, the respective skinned fibre was collected. 10 of each cuff, skinned fibre and whole fibre were isolated and stored in 20µl of membrane preserving buffer (10 mM $NaHCO_3$, 0.25 M sucrose) at -80°C until protein analysis. For Western blotting, 10µl of 3X Laemmli buffer was added to each micro centrifuge tube containing sarcolemmal cuffs, skinned fibres and whole fibres followed by three freeze thaw cycles. Samples were then homogenized with a micro centrifuge tube plunger and spun down before loading.

Antibodies

The antibodies used for this study have been previously used in our lab (25, 41, 42) and other laboratories (2, 13, 17, 53) and are as follows: PLIN3, (Anti-Tip47 (NT) rabbit polyclonal, ProSci Incorporated, #3883, CA), PLIN2 (Adipophilin/ADRP mouse monoclonal, Progen, #610102, Heidlberg, Germany), PLIN5 (Anti-OXPAT, guinea pig polyclonal, Progen, #GP31, Heidlberg, Germany), cytochrome c oxidase complex 4 (COX IV) (COX4 subunit 4, mouse monoclonal, MitoScience, #MS407, TO), Beta-dystroglycan (mouse monoclonal, Abcam, #ab49515, Cambridge, MA), SERCA1 (Anti-SERCA1 ATPase, mouse monoclonal, Thermoscientific, #MA3-911, Rockford, IL), SERCA2 (Anti-SERCA2 ATPase, mouse monoclonal, Abcam, #ab2861, Cambridge, MA).

Western blotting

Protein concentration was determined by Bradford assay (Bio-Rad Protein Assay Dye Reagent Concentrate; #500-0006; Bio-Rad, Mississauga, ON, Canada); red

gastrocnemius immunoprecipitated muscle (n= 9) and mitochondria samples (n= 15) were prepared with a 3X Laemmli buffer and boiled for 5 minutes (except for PLIN5) before loading. An 8% (PLIN3) or 10% (PLIN2, PLIN5 SERCA1, SERCA2, beta-dystroglycan and COX IV) gel was made for protein separation for ~80 minutes at 120V. Proteins were transferred onto polyvinlidene difluride membranes at 100V for 60 minutes. All membranes were cut at the ~25kDa marker and probed for COX IV (10kDa) to quantify as a loading control. Membranes were blocked with 5% fat-free milk (PLIN2, PLIN5, SERCA1, SERCA2, beta-dystroglycan, and COX IV) or 5% bovine serum albumin (BSA) (PLIN3) and diluted with the appropriate primary antibody (1:1000 for PLIN2, PLIN3, PLIN5, SERCA1, SERCA2; 1:500 for beta-dystroglycan; 2:5000 for COX IV) with 5% milk (COX IV), 3% milk (PLIN2, PLIN5, SERCA1, SERCA2, beta-dystroglycan) or 1% BSA (PLIN3) overnight. All membranes were washed in TBST 3 times for 5 minutes and then incubated with secondary antibodies conjugated with horseradish peroxidase (1:10 000 for all proteins except for COX IV which was 1:20 000) diluted in 5% milk (COX IV), 3% milk (PLIN2, PLIN5, SERCA1, SERCA2, beta-dystroglycan) or 1% BSA (PLIN3) for 1 hour. All membranes were washed with TBST 3 times for 10 min and visualized with Chemiluminescent horseradish peroxidase reagent substrate (Peroxide solution + Luminol reagent) (Amersham Biosciences, Piscataway, New Jersey). Images were analysed using Image J software (http://rsbweb.nih.gov/ij/).

Determination of PLIN3-PLIN5 protein interaction

Snap frozen red gastrocnemius muscles (n= 9) were homogenized with 20 times the volume of Griffin lysis buffer (150mM NaCl, 50mM Tris HCl, 1mM EGTA) with protease (1183617001; Roche Diagnostics, Lava, OC, Canada) and phosphatase

(0.406845001; Roche Diagnostics, Lava, QC, Canada) inhibitor tablets. Protein concentration was determined using a Bradford Assay. 1000µg of protein from muscle homogenates were suspended in covalent resin-to-antibody complexes made with Pierce Co-Immunoprecipitation (Co-IP) kit (Thermo Scientific, #26149) and PLIN5 primary anti-body to immobilize PLIN5 protein. Appendix A5 outlines the steps taken to optimize PLIN3 and PLIN5 protein immobilization and quantification. Each sample underwent Western blotting as described above to confirm PLIN5 protein (~52kDa) was immunoprecipitated. Each membrane was then stripped and re-probed with Restore™ Plus Western blot stripping buffer (Thermo Scientific, #46430) and probed for PLIN3 (47kDa). PLIN3 content was made relative to precipitated PLIN5 content.

Statistical Analysis

Total lipid content was measured in rested and contracted samples with a paired one-tailed t-test. Protein content in mitochondria extracted from rest and contracted muscles were normalized to amount of COX IV loaded per lane and analyzed with a paired one-tailed t-test (GraphPad Prism; La Jolla, CA). PLIN5-to-PLIN3 interaction was normalized to precipitated PLIN5 protein with a two-tailed paired t-test. Western blot band density was analyzed with ImageJ software (http://rsbweb.nih.gov/ij.). Statistical significance for all tests was accepted at $p<0.05$.

Results

Neutral lipid utilization during electrically stimulated contraction.

Stimulated contraction caused ~50% reduction in intramuscular lipid content when compared to rested red gastrocnemius muscle (rest, 0.63±0.04% area lipid stained; contracted, 0.32±0.11% area lipid stained, $p=0.03$) (Figure 1).

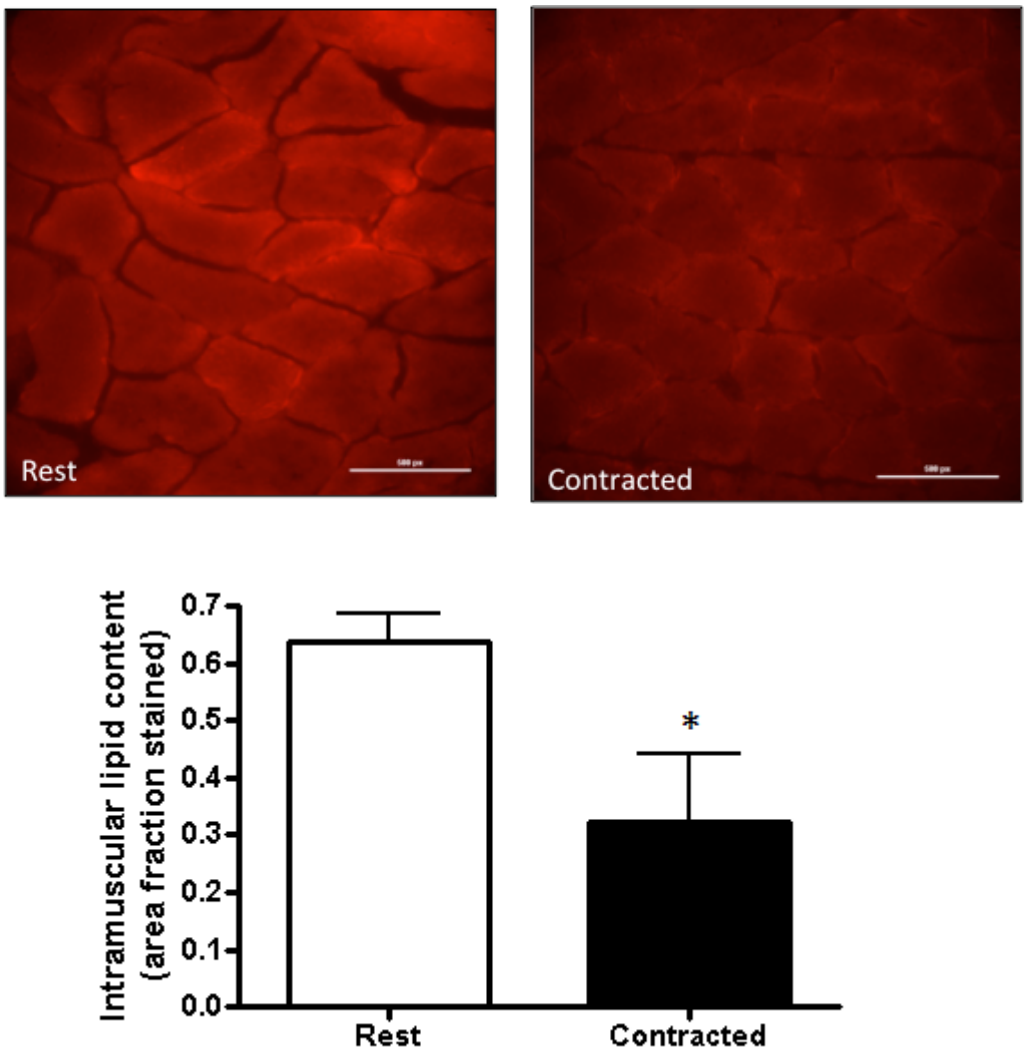

Figure 1. (A) Oil red O immunofluorescent staining of rest and contracted red gastrocnemius muscle (n= 11). Images of one single field of view of muscle cross section. (B) Lipid droplet content (area fraction stained) at rest and following stimulated contraction. Values are expressed as mean ± standard error. Lipid droplet content is significantly lower in contracted muscles when compared to rested muscle (P= 0.03). * Denotes significance accepted at (p> 0.05).

Mitochondria contamination.

There was no detectable contamination of lipid droplets (PLIN2) or sarco(endo)plasmic reticulum (SERCA1 and SERCA2) in purified red gastrocnemius mitochondrial samples (Figure 2). Contrary to the literature (16, 32, 59, 65), there was some sarcolemmal contamination (beta-dystroglycan) present in the purified red gastrocnemius mitochondria with no significant difference from rest to contraction ($p=0.62$). However, PLIN3 and PLIN5 were not found in plantaris sarcolemmal cuffs (Figure 3), nor was there enrichment in the sarcolemma with contraction.

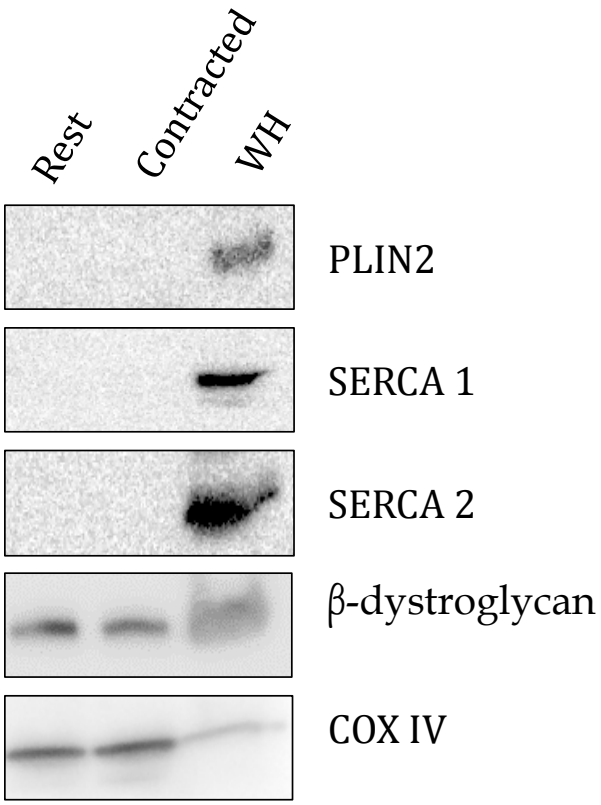

Figure 2. Representative Western blot of rested and contracted mitochondrial samples probing for proteins from different intracellular as follows (n= 15); loading 15µg of mitochondrial protein for PLIN2 (lipid droplets), 20µg of mitochondrial protein for SERCA 1 and 10µg of mitochondrial protein for SERCA 2 (endo(sarco)plasmic reticulum) and 10µg of mitochondrial protein for beta-dystroglycan (sarcolemma) and whole homogenate (WH) with COX IV from corresponding membranes. PLIN2, SERCA1 and SERCA 2 are undetectable in mitochondria samples loaded. Beta-dystroglycan was detected in purified mitochondria samples with no significant differences between rest and contraction (p= 0.62).

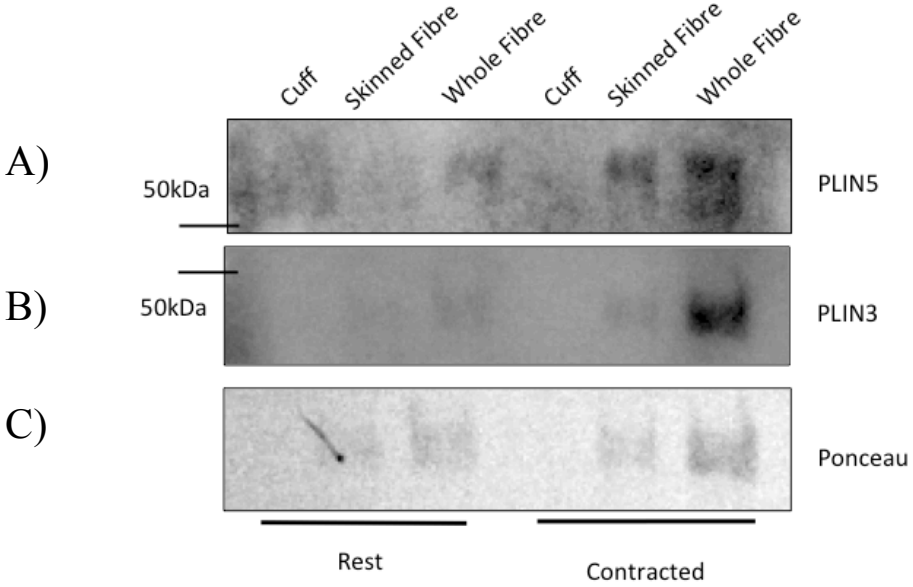

Figure 3. Representative Western blot of sarcolemma cuffs, skinned fibre and whole fibre of rested and contracted plantaris muscle (n= 5) to confirm that PLIN3 and PLIN5 are not detectable. (A) Sarcolemmal cuff, skinned fibre and whole fibre probed PLIN5. (B) Sarcolemmal cuff, skinned fibre and whole fibre probed for PLIN3. (C) Ponceau S stain for total protein

PLIN5 protein content in mitochondria.

PLIN5 content was ~1.6-fold higher in mitochondria isolated from the lipolytically stimulated versus unstimulated red gastrocnemius mitochondria ($p= 0.009$) (Figure 4).

PLIN3 protein content in mitochondria.

With lipolytic contraction, red gastrocnemius mitochondrial PLIN3 protein content was unchanged ($p= 0.21$) (Figure 4).

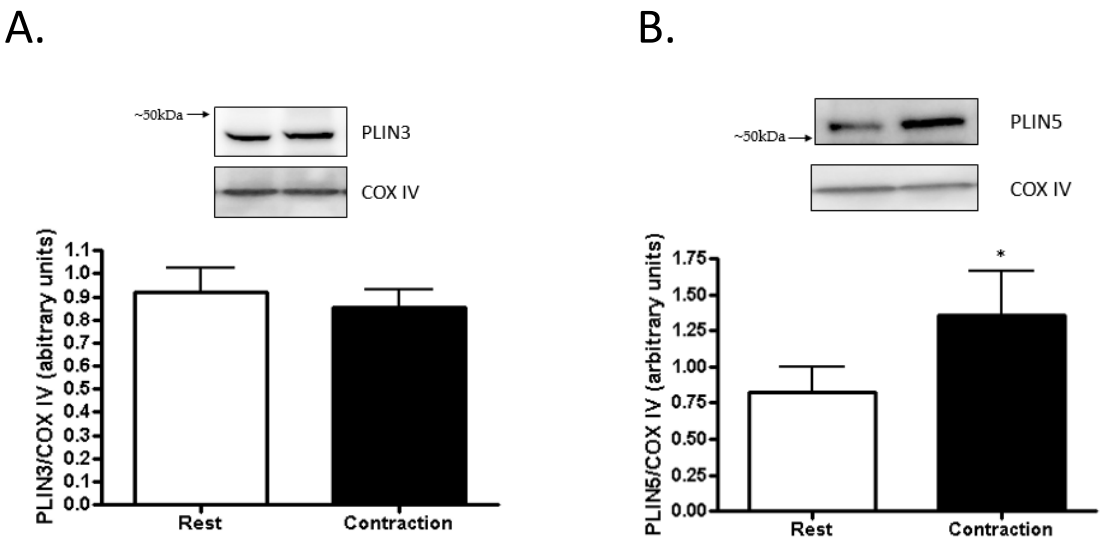

Figure 4. PLIN3 (A) and PLIN5 (B) protein content in rested and contracted skeletal muscle mitochondria (n= 15). (A) Representative Western blot of PLIN3 at rest; *lane 1* and following stimulated contraction*; lane 2*. No significant difference in PLIN3 association with isolated mitochondria following contraction (p= 0.21). (B) Representative Western blot of PLIN5 at rest; *lane 1* and following contraction; *lane 2*. 64% increase in PLIN5 content in isolated mitochondria following contraction (p= 0.009).

PLIN3 and PLIN5 protein-protein interaction

A PLIN3-PLIN5 interaction was measured at rest in whole red gastrocnemius homogenate. This interaction did not change following stimulated contraction ($p= 0.65$) (Figure 5).

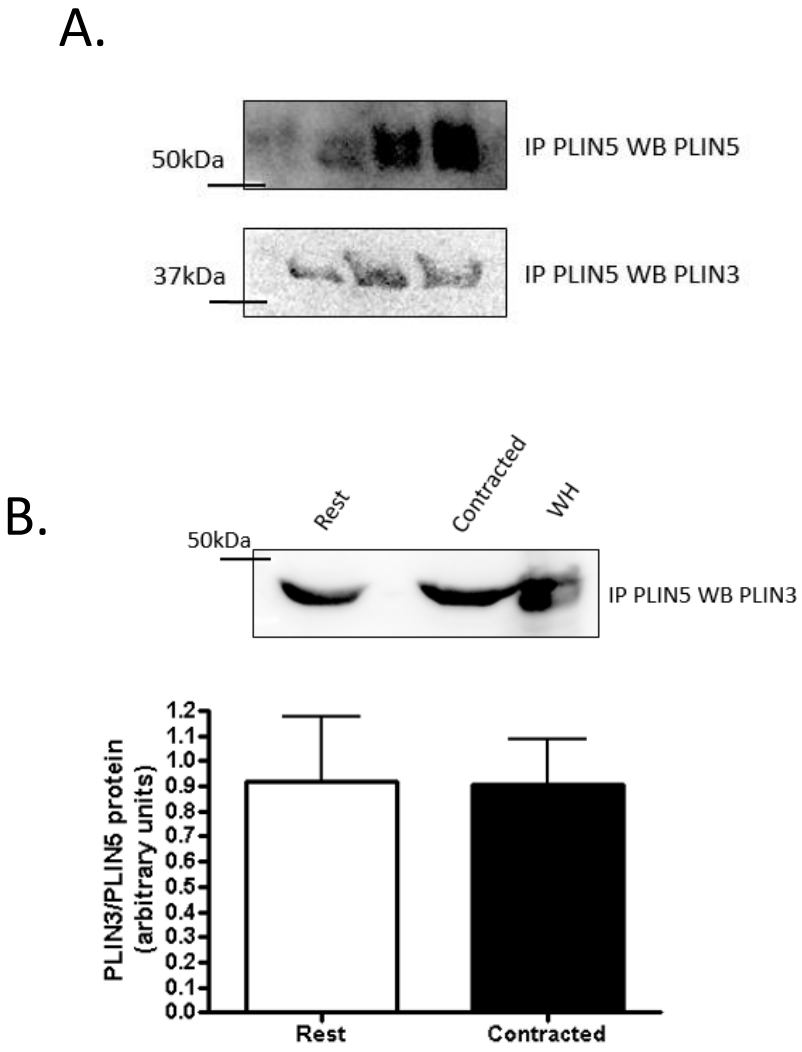

Figure 5. Representative Western blots of the interaction between PLIN3 and PLIN5 (n= 9). (A) Precipitated PLIN5 from red gastrocnemius whole homogenate samples, Western blotted for PLIN5 showing up at approximately 52 kDa loading 10µg (first lane) 15µg (second lane) and 20µg (third lane) of protein. Membrane from stripped and re-probed for PLIN3 showing up at approximately 47 kDa. (B) Representative Western blot of immunoprecipitate PLIN5 samples probed for PLIN3. Graph depicting no change in the interaction between PLIN3 and PLIN5 at rest and following contraction (p= 0.65). IP: immunoprecipitate, WB: Western blot, WH: whole homogenate

Discussion

This study is the first to demonstrate that mitochondrial PLIN5 content in skeletal muscle is increased after 30 min of electrically stimulated contraction that induced lipolysis. Since PLIN5 is higher in the mitochondrial fraction with lipolytic contraction, it is consistent with a role for PLIN5 in coordinating the release of fatty acids from the lipid droplet and their subsequent oxidation in the mitochondria (13, 36, 61). Other novel findings are that PLIN3 protein can be detected in skeletal muscle mitochondria, and the net mitochondrial PLIN3 protein content remained unchanged following contraction. Evidence from the literature would suggest that PLIN3 and PLIN5 are important players in skeletal muscle lipid droplet metabolism (8, 42, 43). Our data adds to the emerging story that PLIN3 and PLIN5 appear to have a role by associating with not only lipid droplets, but also with mitochondria.

The in-vivo sciatic nerve stimulation model used in this study provided the opportunity to pair each contracted muscle to its own resting control, in addition to maintaining physiological conditions during stimulation. This stimulation protocol has been previously used to elicit intracellular fatty acid mobilization (27, 58) although this is the first study to actually report that there is an almost 50% reduction in the amount of muscle lipids following contraction. This confirms that this is an appropriate model to study muscle lipolysis for energy provision during contraction. This reduction in muscle lipid content corroborates other studies using *in vivo* sciatic nerve and isolated soleus stimulation (24, 41, 42, 57).

PLIN5 and PLIN3 protein in skeletal muscle mitochondrial extracts

Our study is the first to demonstrate PLIN5 enrichment in skeletal muscle mitochondria following 30 min of in-vivo stimulated contraction. This suggests a role for PLIN5 in the lipid droplet-to-mitochondria communication that is essential for muscle lipolysis and subsequent oxidation. PLIN5 is widely known for its involvement in fat storage and oxidation as its tissue expression is positively correlated with tissue oxidative capacity (44, 49, 62). Unlike adipose tissue, fatty acids released from skeletal muscle lipid droplets are shuttled directly to mitochondria for oxidation (30, 31). Our results suggest that PLIN5 is perhaps involved in the process of shuttling these fatty acids to the mitochondria due to the robust increase in mitochondrial PLIN5 content after only 30 min of stimulated contraction. This contraction protocol elicited an approximate 50% reduction in neutral lipids demonstrating that a large amount of triglycerides were broken down which would then require a chaperone or conduit to bring them to the mitochondria for oxidation. In addition, Shepherd et al., (55) demonstrated that lipid droplets coated with PLIN5 were preferentially used over lipid droplets not coated with PLIN5 in vastus lateralis collected from sedentary males following both sprint interval and endurance training. Although the exact intracellular location of PLIN5 is not clear, we assume that PLIN5 is chaperoning or directing fatty acids liberated from lipid droplets to the mitochondria during energy requiring situations. PLIN5 protein is not quantifiable in tissues with low mitochondria content (e.g., adipose tissue) (9, 18, 62, 64) and is the most abundant PLIN in tissue that heavily relies on fat oxidation (e.g., cardiac and skeletal muscle), which is consistent with the proposed function for PLIN5 (12, 13, 61). Further research is needed to locate the exact intracellular location of PLIN5 to determine if this mitochondrial enrichment is due to involvement of PLIN5 in regulating the fate of fatty

acids during lipolytic contraction, whether they are hydrolyzed from lipid droplets or recruited from the cytosol to the mitochondria.

This is the first study to indicate that PLIN3 is also found in skeletal muscle mitochondria, and contrary to our hypothesis, this content was unaltered with muscle contraction that induced lipolysis. Due to its ubiquitous expression throughout different tissues (62) and interactions with different intracellular organelles (28, 56), PLIN3 is thought to have many different functions within many different cell types, including; lipogenesis, lipolysis, and trafficking (2, 14, 29, 42, 56, 63). A discrete function for PLIN3 in skeletal muscle has yet to be determined. Prats et al., (50) measured no net change in PLIN3 localization to the lipid droplet with lipolytic stimulation in solei from male Wistar rats, and our results also show no net change in PLIN3 content in red gastrocnemius mitochondria. From this data we can speculate that PLIN3 may act as a type of trafficking protein, regulating the placement of proteins and/or organelle in specific position within the cell and moving them when needed. Therefore, this would require a certain amount of PLIN3 to be present on the lipid droplet and mitochondria to mediate this interaction. This would also be consistent with the proposed role of PLIN3 in intracellular trafficking and the mannose-6-receptor. Here, PLIN3 functions as an effector protein for Rab9-GTPase, recruiting this trafficking protein to the receptor complex where it can initiate the movement of the mannose-6-receptor containing vesicles throughout the cell (2, 6, 22). It is thought that with an acute moderate intensity exercise bout and endurance training, skeletal muscle relies on fatty acids for fuel (19). In addition, after acute exercise and endurance training the association between mitochondria and lipid droplets significantly increases (3, 21, 60). PLIN3 may be a

protein involved in mediating this interaction as it was originally discovered being involved in vesicular transportation (2, 22). The potential mechanism of action requires further exploration and the potential involvement of Rab proteins that associate with lipid droplets and mitochondria.

PLIN3-PLIN5 protein interaction

In addition to the novel findings of the involvement of PLIN3 and PLIN5 with the mitochondria, using co-immunoprecipitation methods we found an interaction between PLIN3 and PLIN5. As expected, this interaction did not change following 30 min of stimulated contraction. While we cannot rule out the possibility of a protein-lipid-protein interaction involving PLIN3 and PLIN5 as suggested previously (8), these two proteins might be working together, as all lipases and proteins seem to be in close proximity to each other in skeletal muscle. We observed no net change in PLIN3 content in mitochondria, but we cannot discount the possibility that PLIN3 is moving back and forth from the lipid droplet to the mitochondria, leaving a consistent amount of PLIN3 at the mitochondria. Previous work from our laboratory identified protein-protein interactions with PLIN3 and PLIN5 individually with CGI-58, ATGL, and HSL under rest, stimulated contraction, epinephrine stimulation and the combination of epinephrine and contraction in skeletal muscle (42, 43). It is likely that these PLIN proteins work together in a complex form to regulate lipid droplet dynamics.

Mitochondrial purity and sarcolemmal cuff analysis

Using a large lower limb muscle that is mixed and oxidative (red gastrocnemius; predominately type I: 51% population, and IIA: 35% population) (20) allowed us to

perform all three protein and lipid analysis using the same muscle. Thus, our experimental approach served to minimize individual variation among rats and to focus on a single muscle for all measurements, except for analysis of sarcolemmal cuffs. Sarcolemmal cuffs were collected from the plantaris muscle because it is a fusiform muscle that allows us to perform the mechanical isolation of the cuffs. The soleus and extensor digitorum longus muscles are also fusiform muscles, but the fibre type populations are at the two extremes (soleus; predominately type I: 84% population and extensor digitorum longus; predominantly type IID/X: 38% and IIB: 38% population) (20), which would make it difficult to interpret the results to a more mixed fibre type like the red gastrocnemius muscle. However, as it is a different muscle, we cannot exclude the possibility of enrichment of PLIN3 and/or PLIN5 in the red gastrocnemius sarcolemma following contraction.

To ensure that the mitochondrial extracts were free from contamination of other intracellular structures, samples underwent a Percoll® gradient for purification and probed for markers of intracellular organelles. Our results suggest that there is no contamination from lipid droplets and sarco(endo)plasmic reticulum which might be expected to contain either PLIN3 or PLIN5, as they have been shown to interact with other intracellular structures in cell culture models (8, 29, 56). There was a measurable contamination of a sarcolemmal marker, beta-dystroglycan, in our purified mitochondria, which is contrary to previous work using the Na^+/K^+ ATPase as a marker of sarcolemmal contamination (16, 32, 58, 65). Primarily subsarcolemmal mitochondria were isolated for these experiments using differential centrifugation, which might not be the gentlest method of isolation, so we might expect to have sarcolemmal contamination as the

mitochondria and sarcolemma are in close proximity in skeletal muscle (10, 23, 54). In addition, an appropriate marker for sarcolemma has yet to be identified (25). However, this is likely not a concern for our study because we were not able to detect any PLIN3 or PLIN5 content in isolated sarcolemmal cuffs either at rest or with contraction. Therefore, this contamination would not be expected to alter the content of either PLIN3 or PLIN5 in our mitochondrial fraction.

Conclusions

Mitochondrial PLIN5 content is increased during contraction, indicating a potential role for PLIN5 in communication between the fatty acids released from lipid droplets and their subsequent oxidation in the mitochondria. It is novel that PLIN3 was detected in skeletal muscle mitochondria, however the role for PLIN3 remains elusive. These data supports the idea of PLIN3 and PLIN5 working together to regulate lipid droplet metabolism by the protein-protein interactions with each other. Understanding PLIN protein function in skeletal muscle is crucial in elucidating their function as intricate regulators of lipolysis in skeletal muscle lipid metabolism.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors

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Chapter 5

Study 2

Changes in rat skeletal muscle mitochondrial PLIN3 and PLIN5 protein content following 8-weeks of endurance training and acute electrically stimulated contraction

S.V. Ramos[1, 2], P.C. Turnbull[1, 2], R.E.K. MacPherson[1], P.J. LeBlanc[1, 3], W.E. Ward[1, 2,3], S.J. Peters[1, 2]

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Affiliations

[1]Center for Bone and Muscle Health, [2]Department of Kinesiology, [3]Department of Health Sciences, Brock University, 500 Glenridge Avenue, St Catharines, ON, L2S 3A1

Contributions:

Sofhia Ramos- Helped to conceive and designed the study, assisted with the endurance training, performed the experiments and mitochondrial extractions, did the biochemical analysis, analyzed and plotted data, wrote manuscript.
Patrick Turnbull- Conceived and designed/supervised the training study, provided intellectual input, edited manuscript drafts, approved final manuscript.
Rebecca MacPherson- Helped to design the study, assisted in developing the model, edited manuscript drafts, approved final manuscript.
Paul LeBlanc- Assisted with the design of the study, assisted with biochemical analysis, edited manuscript drafts, approved final manuscript.
Wendy Ward- Assisted with the design of the study, provided intellectual input, edited manuscript drafts, approved final manuscript.
Sandra Peters – Academic supervisor, helped to conceive and design the study, edited manuscript drafts, approved final manuscript, corresponding author.

Abstract

In skeletal muscle, the association between lipid droplets and mitochondria is increased following endurance training. In addition, skeletal muscle is thought to rely predominantly on intramuscular fatty acids as a fuel following training. Recently, our laboratory examined the association of the lipid droplet-associated proteins PLIN3 and PLIN5 in skeletal muscle mitochondria at rest and following stimulated contraction to induce lipolysis and found an increase in mitochondrial PLIN5 protein content but not PLIN3 following 30 min of contraction. To determine whether PLIN3 and PLIN5 mitochondrial content is altered with endurance training, Sprague Dawley rats (n= 9, age= 51-53 days, weight= 562±18g) underwent 8-weeks of treadmill running (25m/min, 10% incline, 60min) followed by 30min of sciatic nerve stimulation of one limb (10ms impulses, 100Hz/3s at 10-20V; train duration 100ms) to induce lipolysis 48h following the last training bout. Plantaris muscles were removed and embedded for lipid analysis by oil red-O stain and red gastrocnemius muscles were removed and prepared for primarily subsarcolemmal mitochondrial isolation. A two-way ANOVA analyzed acute contraction in sedentary and endurance-trained rats. Mitochondrial PLIN3 protein was ~1.5-fold higher in endurance-trained rats when compared to sedentary (main effect, $p < 0.01$), corresponding to increased intramuscular lipid storage (main effect, $p < 0.001$), with mitochondrial PLIN5 content unchanged following endurance training. Acute lipolytic contraction in sedentary and endurance-trained rats resulted in no net change in PLIN3 mitochondrial content and mitochondrial PLIN5 enrichment increased following acute lipolytic contraction (main effect, $p < 0.01$) corresponding to the degree of decreased intramuscular lipid content in sedentary and endurance-trained rats (main effects, $p < 0.001$). These results suggest that PLIN5 has an acute role during muscle contraction,

where PLIN3 may not. However, PLIN3 may still be important in the mitochondria with chronic adaptations

Keywords: perilipin family proteins, lipolysis, Tip47, OXPAT, intramuscular lipid

Purpose

Outlined in chapter 1 our laboratory made the novel discovery of PLIN3 being associated with skeletal muscle mitochondria with this mitochondrial protein content unchanged following acute contraction. In addition, there was a 65% increase in mitochondrial PLIN5 protein content in contracted hindlimb when compared to the rested limb. In human vastus lateralis muscle following endurance training PLIN3 and PLIN5 protein content is increased in addition to increased mitochondrial and lipid content, leading to the development of the present study. This study aims to determine whether there is a difference in PLIN3 and PLIN5 mitochondrial protein content following acute contraction in sedentary and endurance trained rats.

Hypothesis

1. Isolated subsarcolemmal mitochondria from endurance-trained rats will have a higher PLIN3 and PLIN5 protein content when compared to sedentary rats.
2. Mitochondrial PLIN3 enrichment following acute *in vivo* electrically stimulated muscle contraction will be similar in both sedentary and endurance trained rats.
3. Mitochondrial PLIN5 enrichment following acute in vivo stimulated muscle contraction will be higher in endurance-trained rats when compared to sedentary.

Introduction

The physiological adaptations to endurance training in skeletal muscle are not limited to but include increased oxidative capacity, intramuscular lipid storage, and an increase in the proteins and enzymes involved in regulating oxidative phosphorylation (8, 11). An increased reliance on intramuscular triglycerides as a fuel source after endurance training is seen in some animal (8, 21, 23) and human (12, 26, 38) studies although the methods of measuring intramuscular triglycerides have not been solidified (38, 40). In skeletal muscle, triglycerides are stored in dynamic structures called lipid droplets. Under energy requiring conditions, the triglycerides stored within the lipid droplets can be hydrolyzed by lipases to release fatty acids that are then shuttled to the mitochondria for oxidation and energy production (27, 33). Interestingly, after 8-weeks of endurance training in humans, the distance between lipid droplets and mitochondria significantly decreased in both males and females (37). This would suggest that endurance training elicited a response to increase skeletal muscle efficiency to provide easy access to the mitochondria with fuel for the elevated energy expenditure. Unfortunately, the mechanisms driving this interaction between lipid droplets and mitochondria are currently unknown.

Lipid droplets found within skeletal muscle are composed of a phospholipid monolayer where a host of proteins surround the lipid droplets, regulating lipid droplet metabolism. Of the many proteins surrounding lipid droplets, the perilipin (PLIN1-5) family of proteins is believed to function in lipid droplet synthesis and breakdown (3, 6, 35, 41, 42). PLIN3 and PLIN5 have been implicated in lipid droplet formation in cell culture models (i.e., COS 7, 3T3-L1, cardiomyocytes and CHO cells) but their function in lipid droplet breakdown has yet to be fully defined (7, 16, 41, 42). More recently, our

laboratory measured PLIN5 enrichment in isolated mitochondria from red gastrocnemius muscle following 30 min *in vivo* lipolytic contraction and no change in PLIN3 protein content was found following contraction (Chapter 4). This suggests that these lipid droplet proteins, specifically PLIN5, may play a role in mitochondria during muscle contraction to induce lipolysis, and it is tempting to speculate that they might be involved in the interaction between the lipid droplet and mitochondria during contraction-induced lipolysis. What remains unknown is whether PLIN3 and PLIN5 protein content is altered in mitochondria following endurance training.

PLIN3 and PLIN5 whole muscle protein content increases following endurance and sprint interval training (ranging from 6-12 weeks) in human vastus lateralis muscles (17, 25, 31). Contrary to the increased PLIN3 content following endurance training (17), Peters et al., (25) found no change in PLIN3 content following a 12-week endurance-training program with both males and females that were lean and obese. In addition, many studies have reported a strong correlation between skeletal muscle oxidative capacity following endurance training and PLIN5 content, which suggest that PLIN5 could be closely involved with fatty acid oxidation (25, 42). Although these studies do not report correlations between PLIN3 and PLIN5 and intramuscular lipid content following endurance training (17, 25, 31), it would be expected that PLIN3 and PLIN5 protein content would increase to a similar extent as the increased intramuscular lipid content often seen with endurance training (13, 25). Determining PLIN protein content and subcellular location in response to endurance training may elucidate their involvement in the regulation of lipid storage and use in skeletal muscle.

Whether the mitochondrial content of PLIN3 and PLIN5 protein is altered after endurance training in skeletal muscle has not been investigated. The purpose of this study is to determine if PLIN3 and PLIN5 protein is increased in the mitochondrial fraction of skeletal muscle following an 8-week treadmill running training protocol in rats. In addition, we set out to determine whether there would be a change in the degree of PLIN3 and PLIN5 mitochondrial enrichment following 30 min of *in vivo* electrically stimulated muscle contraction designed to elicit lipolysis when comparing sedentary and endurance trained rats. We expect both PLIN3 and PLIN5 mitochondrial protein to be increased following our endurance training protocol. As seen previously (Chapter 4), we expect PLIN3 mitochondrial enrichment to remain the same following 30 min of lipolytic muscle contraction in both sedentary and endurance trained groups. Also, we expected greater increase in mitochondrial PLIN5 enrichment in endurance-trained rats when compared to sedentary rats following acute contraction.

Methods

Animals

Male Sprague-Dawley rats (n= 20, sedentary: 630 ± 8g endurance trained: 491 ± 10g, age 51-53 days) were purchased from Charles River laboratory (Canada). All procedures and protocols approved by the Animal Care and Utilization Committee at Brock University and conform to all Canadian Council on Animal Care guidelines (22). Rats were housed in pairs; grouping sedentary and endurance trained rats together, in the Comparative Biosciences Facility, maintained on a 12:12 reversed light-dark cycle, fed standard rodent chow, Tekland Global 14% protein, (Harlan Tekland Global, Mississauga, ON, Canada) and had ad libitum access to food and water.

Endurance training and electrically stimulated muscle contraction

Rats were randomly assigned into either sedentary group (n= 10) or endurance trained group (n=10) for 8 weeks. Rats assigned to endurance training began running for 30 min at a speed of 18m/min with a 10% incline and progressed to 60min of running at a 10% incline at a speed of 25m/min. 48 hours after the last exercise bout, rats were anaesthetized with a flow rate of 5% isoflurane and maintained at 3-5% for sciatic nerve stimulation procedures as has been done previously (34)(Chapter 4). A small incision was made on the left leg to expose the sciatic nerve. Curved platinum electrical wires were attached to stimulate the left hind limb to contract for 30 min (10ms impulses, 100Hz/3s at 10-20V; train duration 100ms, two 13 min halves with one 4 min rest) while the right played as an internal resting control. Upon completion, the plantaris muscle was removed and placed in embedding compound cooled in 2-methylbutane and stored at -80°C until further analysis. Red gastrocnemius muscle was also removed and cut in half; one half was prepared for subsarcolemmal mitochondrial isolation while the other half was snap frozen for protein analysis.

Oil red-O staining for intramuscular lipid content

Staining was adapted as done previously (15, 18-20, 30). Briefly, 10µm plantaris muscle sections were sliced at -20°C with a cryotome (ThermoShandon, Runcorn, Cheshire, UK) and placed on microscope slides and kept at -20°C. Slides were thawed at room temperature for ~10 min before fixation in 3.75% formaldehyde. Slides were then washed 3 times for 5 min in deionized water before emersion into a working solution of oil red-O (oil red-O; O0625; Sigma-Aldrich, St. Louis, MO). Slides were washed 3 times for 5 min in deionized water and dried. 10µl of anti-fade reagent (no. P36930; Prolong

Gold Anti-fade Reagent; Invitrogen) was placed on each plantaris muscle section and covered with a glass cover slip. Slides were left to dry in the dark overnight.

Imaging and analysis

Intramuscular lipid content was performed as described previously (15, 18-20, 30). Muscle sections were analyzed using a Nikon Eclipse 80i fluorescence microscope (Nikon Eclipse 80i; Chiyoda-ku, Tokyo, Japan) and capturing images with a digital camera attached to the microscope (Retiga 1300, QImaging, Burnaby, BC, Canada). Images were captured at 40X magnification, with three fields of view/muscle cross-section (insert data) and outlined to determine lipid content within each fibre using imaging software (NIS-Elements AR 3.00; Nikon Instruments, Melville, NY). Oil red-O stain was visualized with 550 fluorophore and lipid content was quantified by manually selecting an intensity threshold that was applied to all images. The number, area and objects within the fibres emitting a fluorescent signal were recorded and expressed as the fraction of the measured area that was stained.

Subsarcolemmal mitochondrial isolation and purification

Primarily subsarcolemmal mitochondria were isolated as described previously (Chapter 4) (24, 34). Briefly, red gastrocnemius muscles were removed and minced on an inverted glass plate on ice and homogenized in 20 times the volume of solution 1 (100mM KCl, 40mM Tris HCl, 10mM Tris base, 5mM $MgSO_4$, 5mM EDTA and 1mM ATP). Samples were spun down at 700g to collect the supernatant and subsequently spun at 14 000g to pellet mitochondria. Mitochondria were washed in 10 times the volume of solution 2 (100mM KCl, 40mM Tris HCl, 5mM Tris base, 1mM MgSO4, .01mM EDTA,

1% BSA and 0.25mM ATP) and 3 (100mM KCl, 40mM Tris HCl, 5mM Tris base, 1mM MgSO4, 0.01mM EDTA, and 0.25mM ATP) for 10 min each at 7000g. Mitochondria were then purified with a 60% Percoll ® gradient before re-suspension in sucrose and mannitol solution (220mM sucrose, 70mM mannitol, 10mM Tris HCl and 0.1mM EDTA). Mitochondria were stored at 80°C until protein analysis.

Purity of the mitochondrial fraction has been previously determined and reported (Chapter 4).

Antibodies

The antibodies used for this study have been previously used in our laboratory and by other laboratories (1, 5, 10, 18-20, 28). PLIN3, (Anti-Tip47 (NT) rabbit polyclonal, ProSci Incorporated, #3883, CA), PLIN5 (Anti-OXPAT, guinea pig polyclonal, Progen, #GP31, Heidlberg, Germany), cytochrome c oxidase complex 4 (COX IV) (COX4 subunit 4, mouse monoclonal, MitoScience, #MS407, TO).

Western blotting

Western blotting procedures were conducted as previously described (10, 19)(Chapter 4) Briefly, protein concentration for mitochondria from red gastrocnemius, was determined by Bradford assay. An 8% (PLIN3 and COXIV) and a 10% (PLIN5) gels were made. Proteins were separated on a polyacrylamide gel for ~80 minutes at 120V and transferred onto a polyviylidene fluoride membrane for 60 minutes at 100V. PLIN3 membranes were cut at the ~25kDa marker and probed for COX IV (10kDa). Membranes were blocked with 5% bovine serum albumin (BSA) (PLIN3), or 5% fat free milk (PLIN5 and COXIV) for one hour before overnight incubation at 4°C with appropriate primary antibody diluted in 1% BSA (1:1000; PLIN3), 3% fat free milk (1:1000; PLIN5)

or 5% fat free milk (2:5000; COXIV). Membranes were washed in TBST for 5 minutes and incubated with secondary antibodies conjugated with horseradish peroxidase in 1% BSA (1:10 000; PLIN3), 3% fat free milk (1:10 000; PLIN5) or 5% fat free milk (1:20 000; COXIV) for 1 hour. Membranes were washed for 10 minutes 3 times and visualized with Chemiluminescent horseradish peroxidase reagent substrate (Peroxide solution + Luminol reagent) (Amersham Biosciences, Piscataway, New Jersey). A Ponceau S stain was used as a loading control for red gastrocnemius of mitochondria and whole homogenates measured. Images were analysed using Image J software (http://rsbweb.nih.gov/ij/).

Statistics

A two-way ANOVA with student-Newman-Keuls post-hoc test was used to determine changes in PLIN3 and PLIN5 protein content in mitochondria samples, whole muscle homogenates and lipid content using Prism statistical software (GraphPad Prism; La Jolla, CA). COXIV protein content in mitochondria samples was analyzed with a one-tailed t-test. Significance was accepted at $p < 0.05$.

Results

Intramuscular triglyceride use following endurance training

Neutral lipid content of endurance-trained rats was ~1.8-fold higher when compared to sedentary rats (main effect, sedentary, 0.25 ± 0.04%, area lipid stained; endurance trained, 0.45 ± 0.01, area lipid stained, p= 0.05) (Figure 1). The sciatic nerve stimulation protocol elicited a 34% decrease in neutral lipid content in sedentary rats (main effect, rest, 0.25 ± 0.04%, area lipid stained; contracted, 0.08 ± 0.01%, area lipid stained, p= 0.002) and a 54% decrease in endurance-trained rats (main effect, rest, 0.45 ± 0.05%, area lipid stained; contracted, 0.25 ± 0.05%, area lipid stained, p= 0.01).

However, the intramuscular lipids used during acute contraction were similar in both groups (Figure 2).

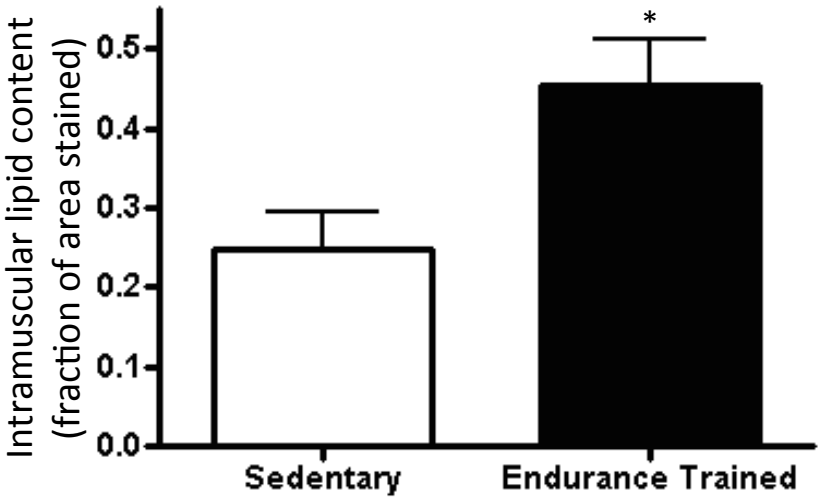

Figure 1. Graph depicting plantaris intramuscular lipid content in sedentary (white bar) and endurance-trained (black bar). Following 8-weeks of training there was a 1.8-fold increase in intramuscular lipids.

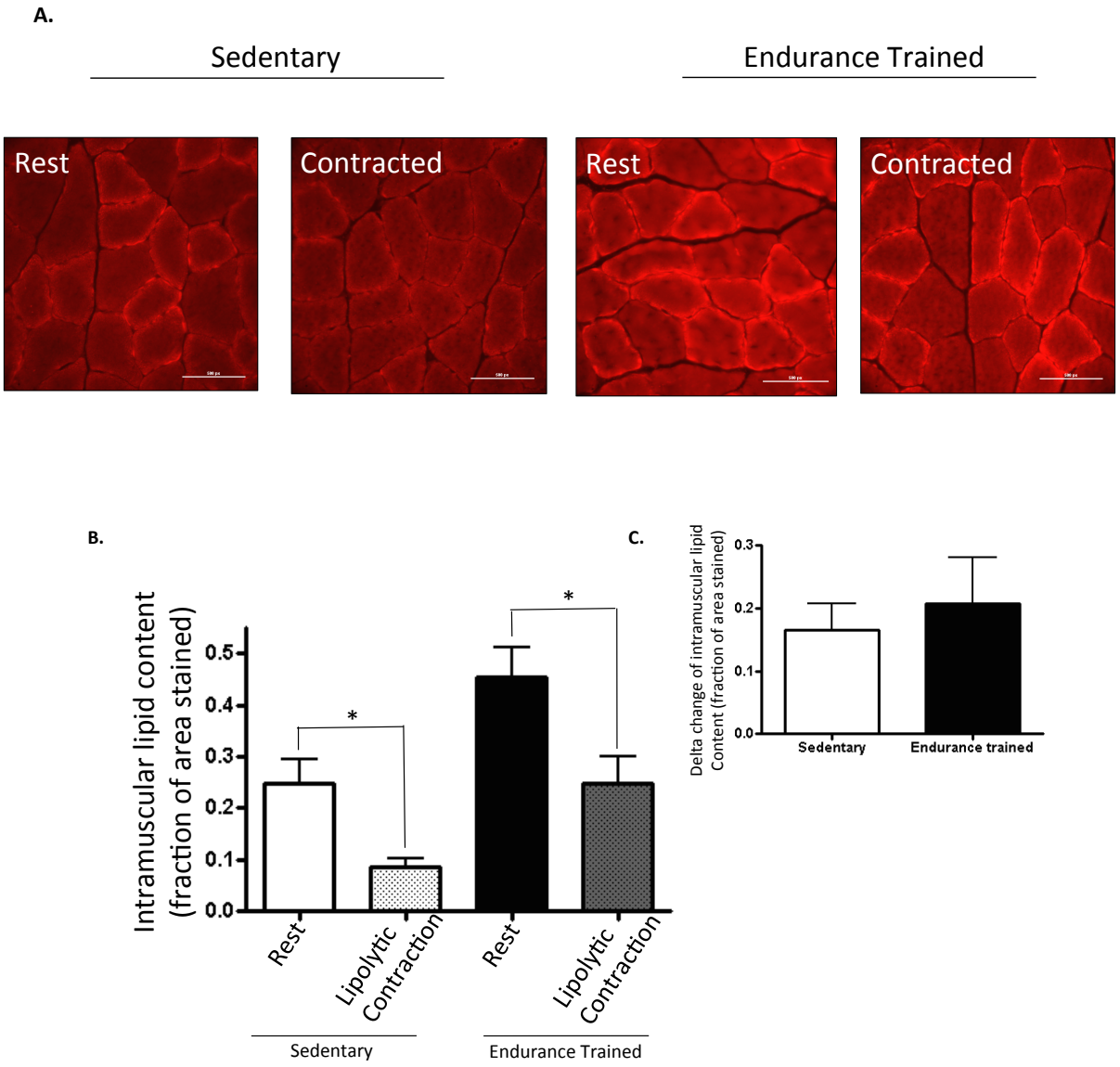

Figure 2. (A) Graphical depiction and (B) visual pictures of intramuscular lipid content in sedentary (light bars) and endurance trained (dark bars) groups. Intramuscular lipid content decreased 32% in sedentary rats and 54% in endurance trained rats. (C) The difference in the amount of intramuscular lipid used during acute contraction is similar in both sedentary and endurance trained groups.

Increased oxidative capacity of skeletal muscle

Skeletal muscle mitochondrial content, measured by COX IV protein content, resulted in a 1.2-fold increase following endurance training ($p= 0.02$) (Figure 3).

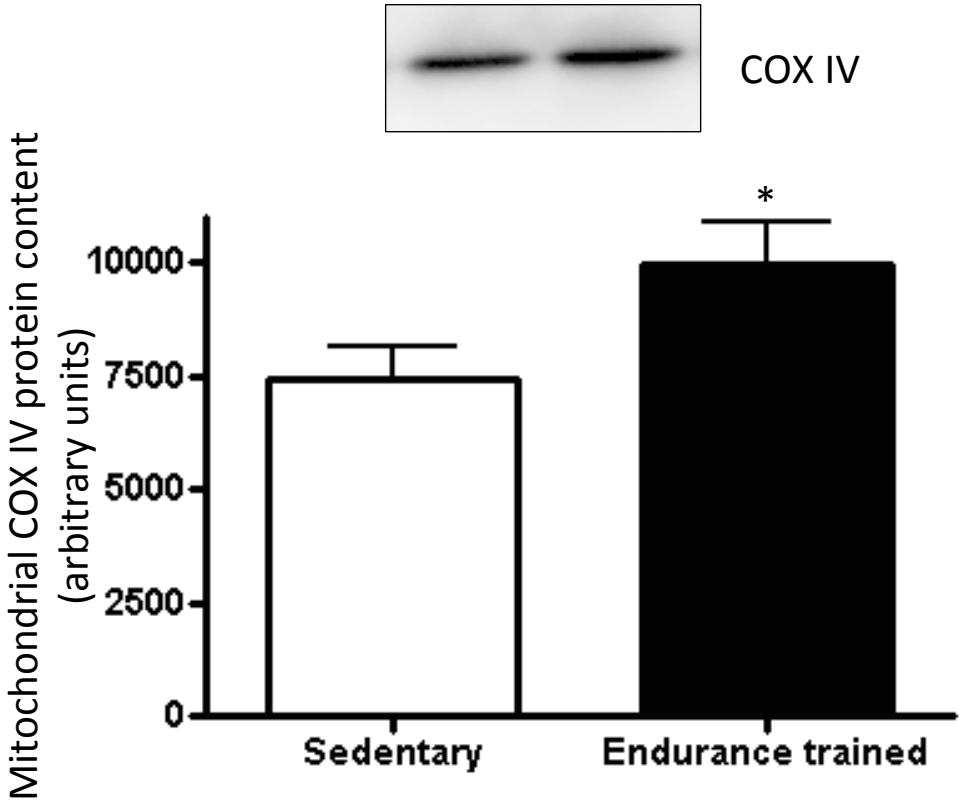

Figure 3. Representative Western blot of COX IV: lane 1, sedentary, lane 2, endurance trained. COX IV protein content is increased ~1.2-fold following endurance training.

Increased PLIN3 protein following endurance training

Mitochondrial PLIN3 content increased ~1.4-fold following endurance training (main effect, p= 0.02) (Figure 4). Following 30 min of lipolytic contraction, PLIN3 mitochondrial and whole muscle protein content is unaltered in both sedentary (p= 0.37) and endurance trained rats (p=0.88) (Figure 4).

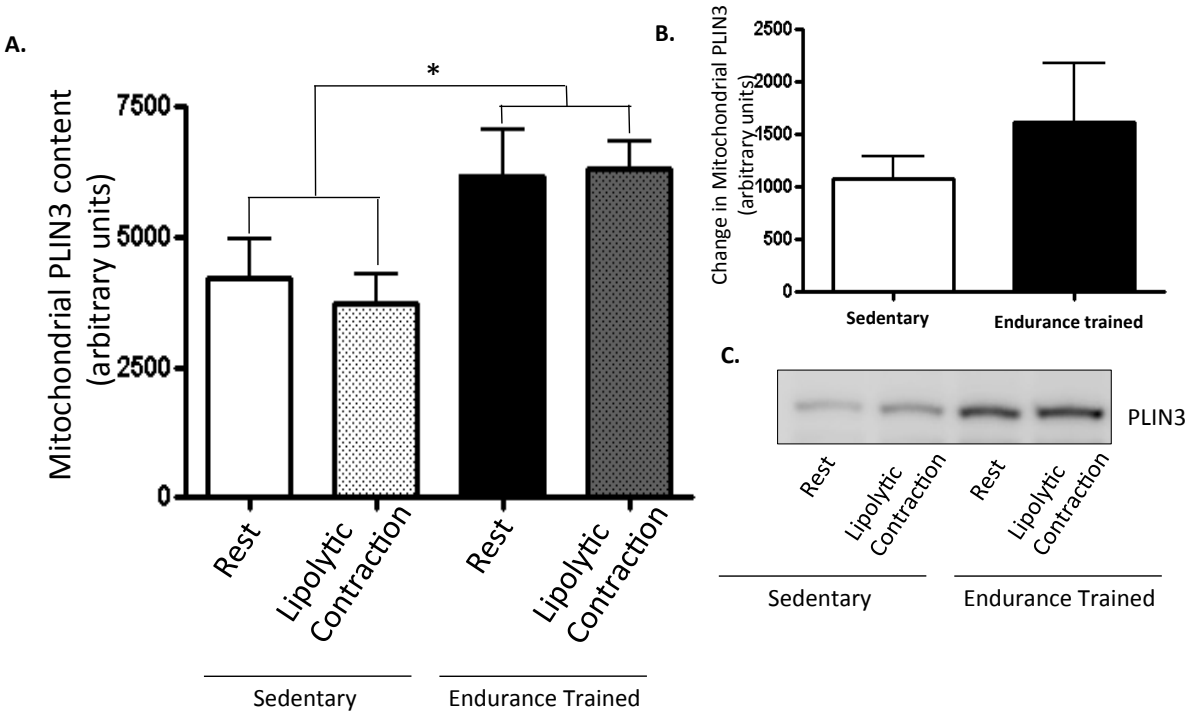

Figure 4. Mitochondrial PLIN3 content in sedentary and endurance trained rats. (A) Graphical depiction mitochondrial PLIN3 content in sedentary (light bars) and endurance trained (dark bars) following acute contraction. (B) Graph depicting no change in mitochondrial PLIN3 enrichment in sedentary and endurance trained rats following acute contraction. (C) Representative Western blot of mitochondrial PLIN3 protein content.

PLIN5 protein content in mitochondria

PLIN5 protein content is unchanged in the mitochondrial fraction following endurance training (p= 0.17) (Figure 5 (A)). PLIN5 protein is enriched to the similar extent in the mitochondrial fraction following 30 min of *in vivo* lipolytic contraction in both sedentary and endurance trained (main effect, sedentary, ~1.6-fold, endurance-trained ~1.7-fold, p= 0.007) rats (Figure 5 (A)). The amount of mitochondrial PLIN5 enrichment is similar following acute contraction in both sedentary and endurance trained rats (p= 0.30) (Figure 5 (B)).

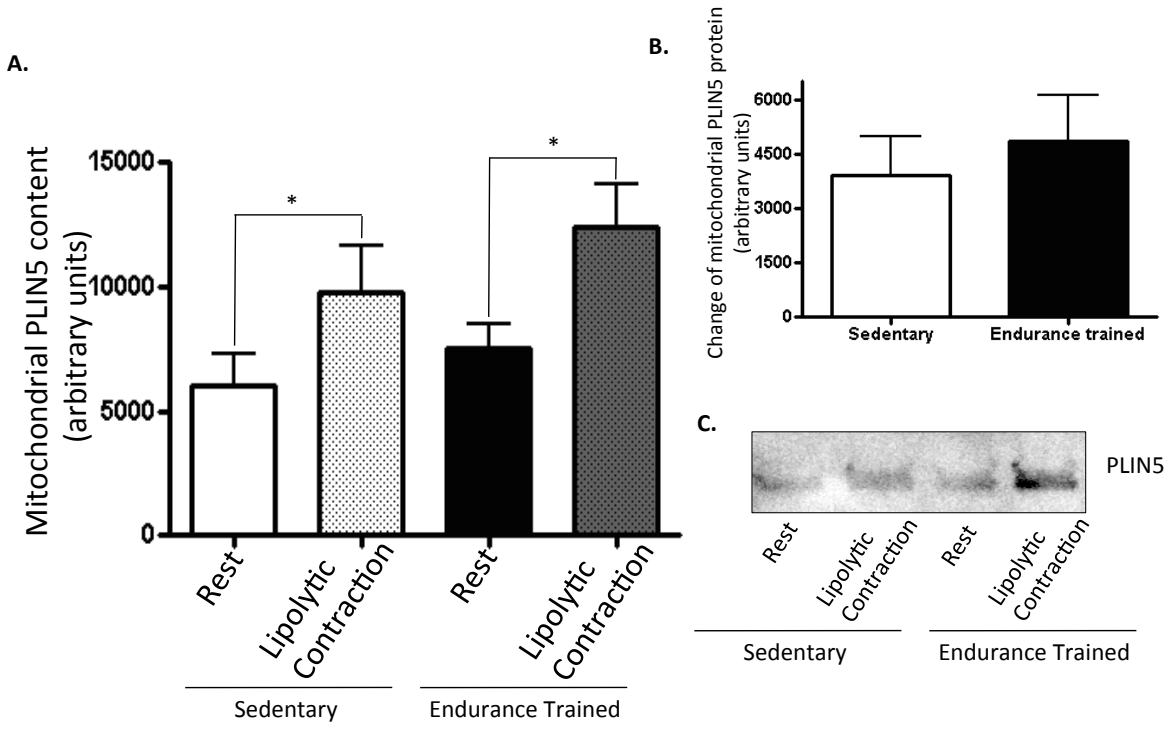

Figure 5. Mitochondrial PLIN5 content in sedentary and endurance trained rats. (A) Graphical depiction mitochondrial PLIN5 content in sedentary (light bars) and endurance trained (dark bars) following acute contraction. (B) Graph depicting no change in mitochondrial PLIN5 enrichment in sedentary and endurance trained rats following acute contraction. (C) Representative Western blot of mitochondrial PLIN3 protein content.

Discussion

This is the first study to look at the lipid droplet associating proteins PLIN3 and PLIN5 and their association with skeletal muscle mitochondria following endurance training, and whether mitochondrial enrichment was altered following acute contraction to induce lipolysis. Following endurance training mitochondrial PLIN3 content increased ~1.4-fold when compared to sedentary rats which is accompanied by an ~1.8-fold increase in intramuscular lipids and an ~1.2-fold mitochondria increase measured by COX IV protein. Interestingly, mitochondrial PLIN5 protein was similar in sedentary and endurance-trained rats. Following acute lipolytic contraction mitochondrial PLIN3 content was unchanged in both sedentary and endurance trained rats as seen in Chapter 4. This would suggest that PLIN3 appears to be important following endurance training but not with acute contraction. Mitochondrial PLIN5 protein following acute lipolytic contraction was increased in both sedentary and endurance trained rats with this increase similar in both groups. Similarly, *in vivo* electrically stimulated contraction to induce lipolysis resulted in a decrease in sedentary (32%) and endurance-trained (54%) rats.

Mitochondrial contamination of other intracellular organelles has previously been documented in our lab (Chapter 4). Briefly, there was no contamination of endoplasmic reticulum or lipid droplets in our purified mitochondrial fraction. Although there was contamination of sarcolemmal membranes in our mitochondria, we have demonstrated that PLIN3 and PLIN5 are not found in resting or contracting sarcolemmal membranes by testing them in isolation (Chapter 4).

Intramuscular triglyceride use following endurance training

Generally, it is believed that following endurance training, there is a greater reliance on intramuscular triglycerides as a primary fuel source during exercise (8, 12, 21,

23, 26, 29, 38). There are limitations in methods for measuring intramuscular triglycerides (e.g. immunofluorescence microscopy, mass resonance spectrometry, electron microscopy, stable isotopes, muscle biopsies with chemical triglyceride extraction). Triglycerides are a very energy dense fuel source where a small decrease in intramuscular lipid content may make a large metabolic contribution. Thus measuring these decreases pose to be quite difficult resulting in considerable variability contributing to the discrepancies found throughout the literature (29, 38). Oil red-O immunofluorescence stain was used to determine change in intramuscular lipid content of sedentary and endurance trained rats at rest and following lipolytic contraction. This staining protocol was selected because it has been shown to be a good method to measure intramuscular lipid content with an intervention model in addition retaining lipid content with formaldehyde fixation (15, 38). Oil red-O stain has been optimized for intramuscular lipid content determination by our (18-20) and other laboratories (15, 30). Our results show that the intramuscular lipids used is similar in both sedentary and endurance trained rats which is consistent with some literature (2, 13, 29) but not others (2, 9, 13, 38).

Endurance trained rats had an almost 2-fold higher intramuscular lipid storage when compared to sedentary rats, which is consistent with the literature (9, 13, 21, 37). In our model, the 30 min *in vivo* sciatic nerve stimulation elicited a decrease in intramuscular lipids in sedentary rats and a greater decrease in endurance trained muscles, but the difference between the two groups did not reach statistical significance (Figure 2). As expected, mitochondrial oxidative capacity, measured by COXIV protein, was increased following endurance training (Figure 3), which, in addition to the increased

intramuscular lipid content, confirming the effectiveness of the treadmill training protocol in promoting the adaptations commonly seen with endurance training (8).

Mitochondrial PLIN3 and PLIN5 protein in endurance trained rats

Our laboratory is the first to identify PLIN3 as the second lipid droplet associated protein to associate with skeletal muscle mitochondria with PLIN3 mitochondrial protein content unchanged following acute stimulated contraction to induce lipolysis (Chapter 4). We were then interested in determining whether this mitochondrial enrichment would be altered following endurance training and found that mitochondrial PLIN3 protein was significantly higher when compared to sedentary rats. Under acute stimulated contraction to induce lipolysis, mitochondrial protein remained unchanged in both sedentary and endurance trained rats, consistent with what was previously done in our lab (Chapter 4). PLIN3 has been implicated as a dynamic protein as it has been reported to interact with various lipolytic proteins under basal and lipolytic conditions in addition to its interactions with other intracellular organelles (1, 19, 20, 32, 36, 43). With endurance training, there is an increased association between lipid droplets and mitochondria in skeletal muscle but how this is mediated is currently unknown (37). Our results suggest that there is a constant amount of PLIN3 associated with the mitochondria at rest and under conditions where lipolysis is stimulated. From this we speculate that PLIN3 may be involved in regulating the placement of the mitochondria under basal and lipolytic conditions. Than and colleagues (36) examined lipid droplet formation and the involvement of Rab40c-GTPase in NRK kidney cells. Here they demonstrated that Rab40c-GTPase is able to interact with PLIN3, potentially aiding in the development of nascent lipid droplets (36). Whether PLIN3 associates with other Rab-GTPase family

proteins to regulate the placement of lipid droplets or mitochondria under conditions where the lipid droplet and mitochondria interact in skeletal muscle requires further investigation.

PLIN5 continues to be a protein of great interest in skeletal muscle lipolysis and fatty acid oxidation, as it seems to play a key role in the interface between lipid droplets and mitochondria. Contrary to our hypothesis mitochondrial PLIN5 protein content was unaltered following endurance training. Endurance training and sprint interval studies in humans have shown increased whole muscle PLIN5 content, but have not characterized mitochondrial PLIN5 protein enrichment in response to training. These results support the idea of PLIN5 not being directly involved in mitochondrial function, as we would have expected to see an increase in PLIN5 mitochondrial protein if this were the case.

It has been speculated that the acute mitochondrial PLIN5 enrichment under lipolytic conditions was a result of PLIN5 playing a role in facilitating or regulating the transfer of newly hydrolyzed fatty acids from lipid droplets to mitochondria (4, 5, 14, 39). Our results support this idea as we continue to see mitochondrial PLIN5 enrichment following 30 min of lipolytic contraction in both sedentary and endurance trained groups, but the amount of enrichment and PLIN5 protein is similar in both groups. However, the amount of mitochondrial enrichment is mirrored by the amount of intramuscular lipids used during the acute lipolytic contraction period where both sedentary and endurance trained rats utilized a similar amount of lipids. Therefore, our results are still consistent with a role for PLIN5 in mitochondria during intramuscular lipolysis, although it is still unclear what this role may be. Future studies should explore different intensities of

contraction where the muscle might rely on intramuscular lipid more or less, potentially altering the degree of mitochondrial PLIN5 enrichment.

PLIN3 and PLIN5 are lipid droplet associating proteins that appear to have important roles not only on the lipid droplet of skeletal muscle but also on the mitochondria. Both these proteins have been implicated in being involved in skeletal muscle lipolysis by their interactions with intracellular lipases and co-lipases (19, 20). Their association with skeletal muscle mitochondria requires further exploration to determine whether this association is due these PLINs regulating fatty acid and/or lipid droplet dynamics.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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Chapter 6

Integrative Discussion

General Discussion

Surrounding the phospholipid monolayer of lipid droplets is the perilipin (PLIN1-5) family of proteins, which are believed to be involved in lipid droplet synthesis and breakdown (5, 7, 8, 23, 24). Of the PLIN family of proteins, PLINs 2-5 are expressed in skeletal muscle where their roles are relatively unknown (5, 42). This thesis focused on PLIN3 and PLIN5 because they are both exchangeable lipid droplet proteins, meaning that they are found on the lipid droplet and in the cytosol, which might suggest that they have a more dynamic function (3).

Our laboratory believed PLIN3 to be a good candidate to be involved in mediating the location of lipid droplets and mitochondria as it was initially discovered by its involvement in vesicular trafficking (1). In addition, the recent discovery of PLIN5 in the mitochondria of skeletal muscle poses the potential for PLIN5 to have a role with the mitochondria during lipolysis. To test this we set out to determine if PLIN3 protein content could be detected in skeletal muscle mitochondria samples. In addition, we aim to determine if mitochondrial PLIN3 and PLIN5 protein was altered following acute lipolytic contraction in both sedentary and endurance trained states.

Summary of Main Findings

Two studies were completed to explore mitochondrial PLIN3 and PLIN5 protein content during stimulated contraction to induce lipolysis under acute contraction and following endurance training in rats. In study 1 (Chapter 4) the novel discovery that PLIN3 protein was abundant in skeletal muscle mitochondria was made, and it was confirmed that PLIN5 could be detected in the mitochondrial fraction. In response to acute lipolytic muscle contraction only PLIN5 protein content increased in the mitochondria. These findings suggest that PLIN5 has an acute role in skeletal muscle

mitochondria during contraction that induces lipolysis. However, PLIN3 content of the mitochondria was not altered with acute contraction, and therefore either it does not have a mitochondrial role in acute situations, or there is sufficient amount of PLIN3 protein in the mitochondria to support its role. In addition, there is an interaction between PLIN3 and PLIN5 at rest and following acute lipolytic contraction, which would suggest that PLIN3 and PLIN5 work together during lipolysis.

Study 2 (Chapter 5) aimed to characterize PLIN3 and PLIN5 mitochondrial enrichment in sedentary and endurance-trained rats after acute contraction that induced lipolysis. Although the literature is variable, endurance training is generally considered a perturbation that would be expected to increase the rate of muscle lipolysis and oxidation (10, 14, 30, 32, 37). Here we found that only PLIN3 and not PLIN5 protein is increased in skeletal muscle mitochondria following endurance training. However, with acute muscle contraction, mitochondrial PLIN5 content was increased similarly in both sedentary and endurance trained rats. A secondary aim of this study was to determine whether endurance trained rats relied more on intramuscular lipids during contraction-induced lipolysis when compared to sedentary rats. Our results demonstrate that with this *in vivo* contraction model, endurance trained and sedentary rats neutral lipid content decreased to a similar extent. Therefore, this still supports a role for PLIN5 in the mitochondrial fraction during acute muscle contraction-induced lipolysis since the drop in muscle lipid content effectively mirrored the increased mitochondrial PLIN5 content. Outlined below are the potential mechanisms by which PLIN3 and PLIN5 may mediate the communication between lipid droplets and mitochondria during lipolysis that is stimulated acutely, and when compared between sedentary and endurance trained rats.

Lipid droplet and mitochondrial movement mediated by PLIN3

More recently, the literature has shifted away from the idea of lipid droplets being static storage depots to recognizing them as dynamic storage organelles. Specifically in skeletal muscle, the lipid droplet plays an important role predominantly storing substrates in the form of triglycerides as a fuel source for the mitochondria (15, 35). With an acute exercise bout and following endurance training, the percentage of lipid droplets touching mitochondria increases, but the mechanism of this remains elusive (11, 41). PLIN3 is a good candidate to mediate this interaction for the following reasons: a) PLIN3 is found both on lipid droplets (33) and associated with the mitochondria in skeletal muscle (Chapter 4); b) in an *ex vivo* soleus preparation, electrically stimulated muscle contraction and epinephrine stimulation to induce lipolysis resulted with no net change in PLIN3 content on lipid droplets (33); c) similarly, with acute *in vivo* contraction of sedentary (rested) and endurance trained rats mitochondrial PLIN3 protein content is unchanged (Chapters 4,5); d) mitochondrial PLIN3 content is significantly increased in endurance trained rats when compared to sedentary and e) PLIN3 was originally discovered with an intracellular trafficking function (1, 9). Cumulatively, we can speculate that a certain amount of PLIN3 is required to be present on lipid droplets and mitochondria (points b and c) to regulate their intracellular placement at rest and during contraction.

Figure 15 depicts a schematic diagram of how this lipid droplet-mitochondrial interaction may ensue with the involvement of PLIN3. At rest, PLIN3 is found on the lipid droplet, in the cytosolic environment (5, 33) and on the mitochondria (Chapter 4 and 5). With an acute bout of electrically stimulated contraction PLIN3 content is unaltered (Figure 15. B). What remains unknown is whether PLIN3 is constantly moving (e.g., from lipid droplet to mitochondria to cytosol) during lipolysis (Figure 15 B and D).

Following endurance training mitochondrial PLIN3 content is ~1.4-fold higher when compared to sedentary rats (Chapter 5). These data suggest that the association between PLIN3 and the mitochondria may be important with endurance training as there was also an increased oxidative capacity as measured by COX IV (~1.2-fold increase) and an increased energy storage in the form of intramuscular lipids (~1.8-fold increase) (Chapter 5)(Figure 15, C). This increased oxidative capacity is thought to be a result of an increased capacity for the muscle to utilize fuel during exercise, which is complemented with an increased storage of fuel in the form of triglycerides. The increased PLIN3 mitochondrial protein may play a role in the dynamics of lipid droplets and mitochondria. As stated above, acute contraction and endurance training increases the interaction between lipid droplets and mitochondria in skeletal muscle (11, 41). The unaltered PLIN3 content may be regulating the distance between lipid droplets and mitochondria as PLIN3 was initially discovered to be involved in intracellular trafficking (1, 9, 12). This is further supported by the unchanged mitochondrial PLIN3 content following acute contraction in both sedentary and endurance trained rats, similar to our results in Chapter 4.

PLIN3 functions as an effector protein in intracellular trafficking, recruiting Rab9-GTPase to the M6PR complex which then allows the intracellular movement of vesicles containing M6PRs (1). In addition to the PLIN family of proteins, the lipid droplet is surrounding by a host of proteins including Rab-GTPase proteins. Currently, there is a known interaction between Rab18-GTPase and PLIN2 on the lipid droplet but the function of this interaction is unknown (34). In NRK epithelial and HeLa cells an interaction between Rab40c-GTPase and PLIN3 was identified on lipid droplets (40).

This report suggests that Rab40c-GTPase is involved in the formation of new lipid droplets. In addition, the Rab-GTPase Ypt11 is known to interact and mediate the movement of the endoplasmic reticulum, golgi apparatus and mitochondria during yeast division (22). As both PLIN2 and PLIN3 have been shown to interact with Rab-GTPases on the lipid droplet, perhaps there is a PLIN3 interaction with another Rab-GTPase on the lipid droplet as described in Figure 16, where our results demonstrate that with contraction there may be a constant amount of PLIN3 on the mitochondria which may be interacting with a Rab-GTPase regulating mitochondrial placement (Chapter 4 and 5). As the lipid droplet-mitochondrial distance decreases with endurance training and acute contraction (11, 41), PLIN3 may also be mediating this interaction as there is an increased mitochondrial PLIN3 content and a known increase in PLIN3 protein in whole muscle following endurance training (25, 38)(Figure 16).

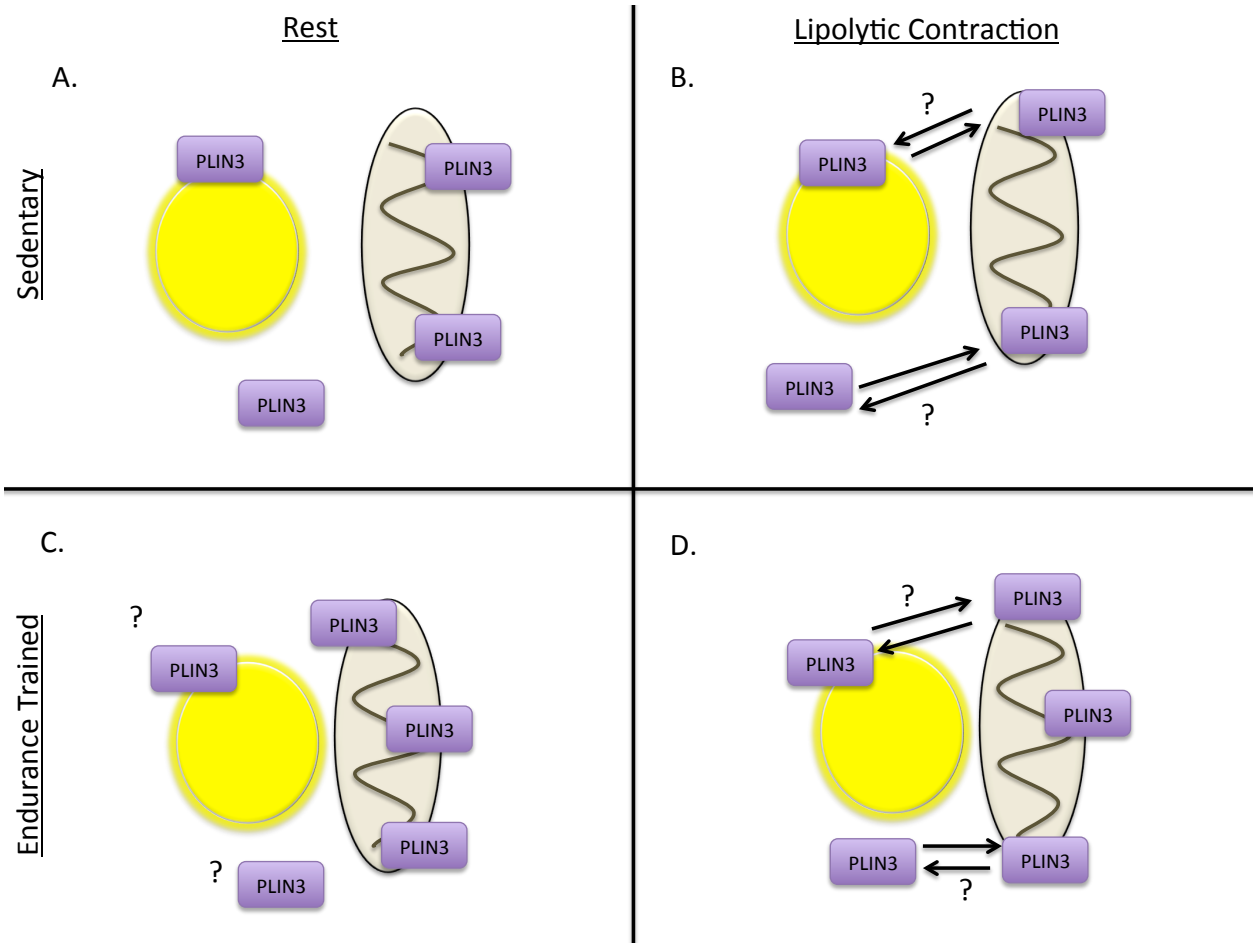

Figure 15. PLIN3 interactions with skeletal muscle lipid droplet and mitochondria under basal and lipolytic contraction conditions in a sedentary and endurance trained state. **A)** Under basal conditions PLIN3 can be found on the lipid droplet, in the cytosolic environment and on the mitochondria. **B)** With acute lipolytic contraction the amount of PLIN3 that co-localises with the lipid droplet remains unchanged (33) similarly to the mitochondrial PLIN3 content. What remains unknown is whether PLIN3 is cycling on and off the lipid droplet and/or mitochondria from the lipid droplet or cytosol. **C)** In endurance-trained rats under basal conditions mitochondrial PLIN3 content is higher when compared to basal sedentary rats (A). Whether lipid droplet bound or cytosolic PLIN3 is altered with endurance training is unknown. **D)** With acute lipolytic contraction in endurance-trained rats, mitochondrial PLIN3 content is unchanged. Whether there is cycling of PLIN3 from the lipid droplet or cytosol to and from the mitochondria is unknown.

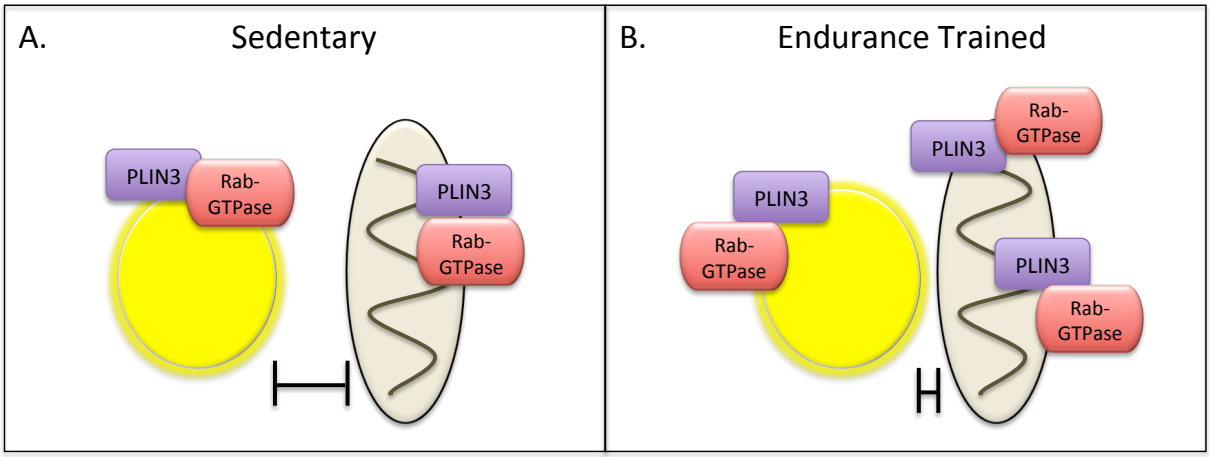

Figure 16. A speculative model of the lipid droplet-mitochondrial interaction in skeletal muscle mediated by PLIN3 and a Rab-GTPase molecule. **A)** In a sedentary state lipid droplets and mitochondria are in close proximity, which is thought to be to mediate the flux of fatty acids to the mitochondria when needed. During lipolytic contraction the amount of PLIN3 on the lipid droplet and mitochondria is unchanged. We can speculate that PLIN3 may interact with Rab-GTPase molecules known to inhabit both lipid droplets and mitochondria, regulating the placement of the lipid droplet and mitochondria. **B)** Following endurance training there is an increased association between lipid droplets and mitochondria in addition to an increased lipid droplet and mitochondrial content. This interaction may be mediated by PLIN3 interacting with Rab-GTPases that are known to interact with lipid droplets and mitochondria regulating the placement of lipid droplets and mitochondria during rest and during times of increased energy demand.

Fate of intramuscular fatty acids regulation by PLIN5

During lipolysis, fatty acids hydrolyzed from lipid droplets are shuttled directly to the mitochondria where they may be oxidized or may be re-esterified into triglycerides on the lipid droplet (36, 39). How fatty acids are selected for oxidation and re-esterification in skeletal muscle is unknown. In addition, newly hydrolyzed fatty acids need to be removed from lipid droplets and transported to the mitochondria where they can be incorporated into a fatty acid-acyl CoA so it can enter the mitochondria for oxidation (35). The results from Chapters 4 and 5 support the role of PLIN5 as the protein that may be involved in the regulating fatty acids that are directed for oxidation to the mitochondria. Under basal (resting) conditions in both sedentary and endurance trained rats there is a similar amount of mitochondrial PLIN5 protein (Chapter 4 and 5) (Figure 18 A and C). If PLIN5 was closely involved with the machinery of the mitochondria we would expect basal mitochondrial PLIN5 protein to increase in a similar manner to the increased mitochondria content and increased intramuscular lipid content often seen with endurance training but we did not. In whole muscle collected from humans, total PLIN5 content increases with endurance training (25, 31, 38) suggesting that PLIN5 may be more involved the movement of fatty acids to the mitochondria than actually mitochondrial function and beta-oxidation.

With acute lipolytic contraction mitochondrial PLIN5 protein significantly increases. Moreover, this acute mitochondrial PLIN5 increase is similar in both sedentary and endurance trained rats. Regardless of training status, lipid and mitochondrial content, the amount of PLIN5 enrichment remained somewhat similar. This suggests that the mitochondrial PLIN5 enrichment maybe due to PLIN5 translocation from lipid droplets to mitochondria. Supporting this theory is work done in our laboratory measuring PLIN5

localization with skeletal muscle lipid droplets following acute *in vitro* stimulated lipolytic contraction (26). Here we measured no net change in PLIN5 co-localization with lipid droplets following lipolytic contraction which could be do to the proportional decrease in lipid droplet density and surface area with a similar decrease in the amount of PLIN5 protein on the lipid droplet resulting in a certain amount of PLIN5 left on the lipid droplet (26). Shepherd et al., 2013 (38) analyzed lipid droplets coated with PLIN5 and found that after an exercise bout in trained individuals, the amount of PLIN5 decreases similarly to the subsequent decrease in lipid droplet density. Perhaps as the lipid droplet becomes smaller by the loss of fatty acids, PLIN5 content is also decreasing as its taking these newly hydrolysed fatty acids to the mitochondria for oxidation, as seen with the increased mitochondrial PLIN5 content in Chapters 4 and 5.

A representative diagram of how this might be occurring is depicted in figure 17 (B.) where during lipolysis stimulated by contraction, fatty acids are being hydrolyzed and re-esterified into triglycerides or shuttled off to the mitochondria for oxidation. PLIN5 may be sequestering fatty acids released from the lipid droplet or from the cytosol and bringing them to the mitochondria where they can then be transported into the mitochondria. We can speculate that this procedure occurs in a similar fashion in endurance trained rats undergoing acute stimulated contraction (Figure 17 D), as the mitochondrial PLIN5 enrichment is similar in both endurance trained and sedentary rats.

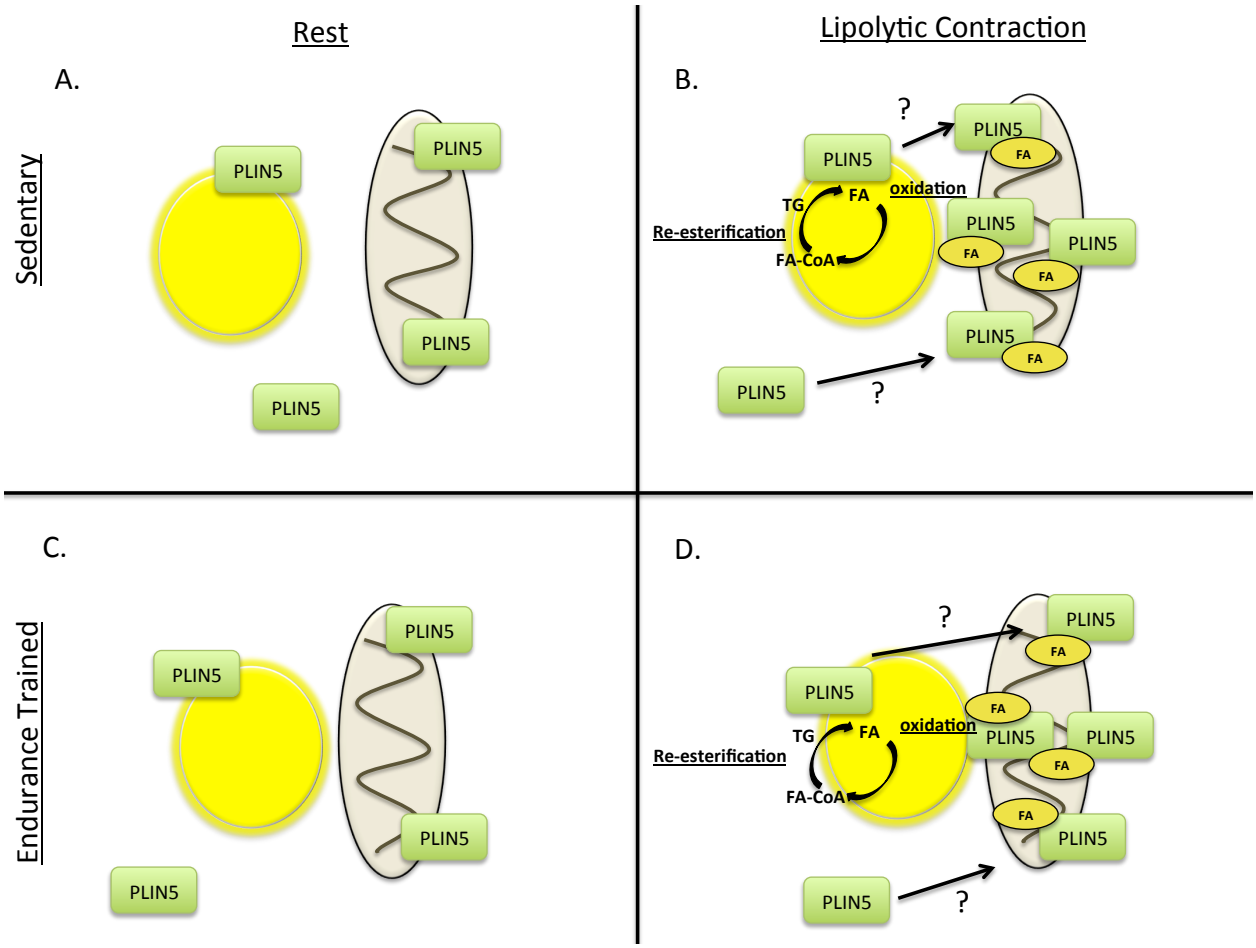

Figure 17. A speculative model for mitochondrial PLIN5 on skeletal muscle lipid droplets and mitochondria under basal and acute lipolytic contraction conditions in sedentary and endurance trained rats. **A)** Under basal conditions PLIN5 is found on the lipid droplet, in the cytosol and on the mitochondria. **B)** With acute lipolytic contraction, PLIN5 is enriched in the mitochondrial fraction. How this enrichment occurs is unknown. PLIN5 may be moving from the lipid droplet as fatty acids are hydrolyzed or may be moving from the cytosol as fatty acids are brought into the muscle. **C)** Under basal conditions in endurance-trained rats, mitochondrial PLIN5 content is similar to basal sedentary rats (A). Whether there is an increased lipid droplet bound or cytosolic PLIN5 content following endurance training is unknown. **D)** Acute lipolytic contraction in endurance trained rats resulting with an increased mitochondrial PLIN5 content similarly to the increase seen in sedentary rats following acute lipolytic contraction. Whether PLIN5 is moving from the lipid droplet or from the cytosol is unknown. Here we can speculate that PLIN5 may be regulating the flux of fatty acids released from lipid droplets or brought into the muscle, as the mitochondrial PLIN5 enrichment is similar in both sedentary and endurance-trained rats.

Conclusions

This thesis has set the foundation for further exploration of the PLIN proteins in skeletal muscle not only with the lipid droplet but also with the mitochondria. Here, we have made the novel discovery that PLIN3 can be found in isolated skeletal muscle mitochondria samples. However, contrary to our earlier hypothesis, with acute stimulated contraction, mitochondrial PLIN3 protein content remains similar in contracted compared to resting conditions. We confirmed in our hands that PLIN5 was detectable in mitochondrial extracts, and consistent with our hypothesis mitochondrial PLIN5 protein increased with acute contraction. In addition to these novel findings, when comparing sedentary and endurance trained rats we reported a higher mitochondrial PLIN3 protein content in endurance trained rat skeletal muscle when compared to sedentary, which was consistent with our hypothesis. However, contrary to our hypothesis, mitochondrial PLIN5 protein was similar in both groups. Also contrary to our hypothesis, following acute contraction mitochondrial PLIN5 protein content increased to a similar extent in both sedentary and endurance trained rats. Consistent with what we observed in the first study, however, mitochondrial PLIN3 protein was unchanged following contraction.

These two PLIN proteins may be involved in skeletal muscle lipolysis, more specifically, it is possible that PLIN5 may be involved in regulating the flux of fatty acids to the mitochondria during contraction induced lipolysis, while PLIN3 may be more important in mitochondria with chronic perturbations such as endurance training. Further work is required on both PLIN proteins to determine a discrete role in skeletal muscle lipid metabolism and the lipid droplet mitochondrial interaction.

Study Strengths and Limitations

The primary outcome of these experiments were to determine whether there was a change in mitochondrial PLIN3 and PLIN5 protein content following 30 min of stimulated contraction to induce lipolysis in both sedentary and endurance trained rats. An animal model using Long Evans (Chapter 4) and Sprague Dawley (Chapter 5) rats was used for each respective experiment. We do not believe that the use of two different strains of rats in the thesis compromises the results (i.e., study 1: Long Evans, study 2: Sprague Dawley). The fiber types of the muscle used are thought to be similar between species and we were able to repeat our results for mitochondrial PLIN3 and PLIN5 content following acute stimulated contraction between the species. For this thesis a rat model was used as it is often seen in muscle physiology research, providing muscles with a more specific fibre type, which allows the discrimination between type I, IIa and IIx/b fibres that is difficult to do in a human model. Moreover, we were able to collect the large red gastrocnemius muscle (~ 250mg), that was divided for histochemical and protein analysis while still having a substantial amount of muscle for mitochondrial isolation. Using a human model we would not have been able to collect the amounts of muscle needed to complete all of the measures for this study.

Chapter 4 used a sedentary animal model where the sciatic nerve was exposed on anaesthetized Long Evans rats to electrically stimulate the muscles of the lower limb to contract. This method of stimulating contraction is a major strength for this study because during the contraction period the muscles used for protein and lipid analysis were kept in its physiological environment, which should provide a better "snap shot" of the state of proteins and enzymes following our manipulation. In addition, this method allowed for the contralateral limb of each rat to be its own internal resting control resulting in a

physiological and statistical strength. Study 2 (Chapter 5) utilized an endurance training protocol adapted from previous treadmill protocols (14, 21). This perturbation was selected as it has been previously been shown to be a good stress to induce the adaptations often seen in human models with endurance training.

The studies completed in this thesis provide novel insights as to how PLIN3 and PLIN5 respond in the mitochondrial fraction of skeletal muscle following acute contraction in sedentary and endurance-trained rats. While there are many strengths to these studies, there are also some limitations. As most physiology research is done in animal models in an attempt to understand physiological mechanisms behind diseases in humans, these studies were completed in animal models therefore we can not infer that our findings occur in the same fashion in human skeletal muscle. In addition, Study 1 (Chapter 4) used two different muscles to analyze PLIN3 and PLIN5 protein content in the sarcolemmal and mitochondria from the plantaris and red gastrocnemius muscle respectively, where we cannot interpret these results to be interchangeable because they are two different muscles. Another limitation to both Study 1 and 2 (Chapter 4 and 5) is that the different fractions of the muscle cell (cytosol, nucleus, mitochondria) were not isolated there for we cannot describe the mitochondrial PLIN5 content in response to acute contraction from PLIN5 movement from either lipid droplet or cytosol to the mitochondria.

Implications of Findings

The progression of the North American diet has resulted in many overweight and obese Canadians and Americans, which has been positively correlated with the development of serious metabolic diseases and even death (4, 6, 13). It was originally

believed that elevated intramuscular lipid content was the underlying cause of the development of these metabolic disorders. Reports on insulin sensitive endurance trained individuals have demonstrated similar intramuscular lipid content to individuals with insulin resistance leading to the development of the "athletes' paradox" where the regulation of the storage and use of intramuscular lipids may underlie the metabolic difference between obese and endurance trained individuals (2). Skeletal muscle of endurance-trained individuals is very well organized with a high lipid and mitochondrial content (2). Endurance training in an obese population results in increased mitochondrial content and insulin sensitivity (17-20), where with the progression of insulin resistance there is an increased lipid content and decreased mitochondrial content (2, 6, 16). Perhaps it is the regulation of both the lipid droplet and its interaction with the mitochondria that contribute to the paradoxical difference between obese and endurance trained populations. This thesis aimed to understand the relationship between lipid droplets and mitochondria by studying the mitochondrial content of lipid droplet associating PLIN proteins under resting and lipolytic conditions in rat skeletal muscle.

Data collected from Chapters 4 and 5 has provided evidence that the interaction between lipid droplets and mitochondria may be important during contraction induced lipolysis in skeletal muscle. Here we have uncovered an *in vivo* physiological response after 30 minutes of stimulated contraction in addition to the novel discovery of a second lipid droplet associating protein associating with the mitochondria. This research has the potential to set the foundation for characterizing potential functions for these PLIN family members as their role in skeletal muscle is currently unknown. Developing a better understanding of how PLIN3 and PLIN5 interact with skeletal muscle lipid

droplets and mitochondria in this "sedentary and endurance trained rat model" we can then apply this knowledge to different metabolic rat populations (e.g., obese insulin resistant or type 2 diabetic) to determine whether there are any changes in how they respond under lipolytic conditions.

Future Directions

PLIN3 localization with lipid droplets following endurance training

Results from this thesis have demonstrated that PLIN3 is associated with skeletal muscle mitochondria and this mitochondrial PLIN3 content is unaltered in both sedentary and endurance-trained rats following acute contraction to induce lipolysis (Chapters 4 and 5). From these results we speculated that PLIN3 might be involved in regulating the placement or movement of mitochondria and potentially lipid droplets as PLIN3 has a known role in vesicular transportation though its interaction with Rab9-GTPase (1, 9). With acute contraction and following endurance training, the distance between lipid droplets and mitochondria decreases, where we can assume, to allow more efficient transport of fatty acids to the mitochondria (11, 41). Here, we demonstrated that under sedentary and endurance-trained conditions following acute contraction, there is a consistent amount of PLIN3 associated with skeletal muscle mitochondria. With acute contraction and epinephrine stimulation in skeletal muscle the amount of PLIN3 co-localized with lipid droplets remains unchanged (33). What remains unknown is whether there is an increased localization of PLIN3 to skeletal muscle lipid droplets following endurance training. Future work should look to determine whether lipid droplet PLIN3 content is altered with endurance training and whether this content is changed with acute contraction. In addition, to determine whether PLIN3 is involved in the movement of lipid droplets and mitochondria during energy requiring situations, research should look

to see if PLIN3 interacts with Rab-GTPase proteins that associate with lipid droplets and mitochondria in skeletal muscle. The Rab-GTPase family of proteins are known intracellular trafficking proteins, with a sub set of this family found surrounding the lipid droplet membrane (Rab 1, 2, 3d, 5, 7, and 18) (29). In addition to those Rab-GTPase proteins, the Rab-GTPase Ypt11 is found on the mitochondrial membrane and is responsible for the movement of the endoplasmic reticulum, golgi apparatus and mitochondria during yeast cell division (22). Whether PLIN3 interacts with these Rab-GTPases on the lipid droplet and mitochondria and whether this is altered with lipolytic contraction has not been investigated.

Regulation of newly hydrolyzed intramuscular fatty acids by PLIN5

Chapters 4 and 5 have shown mitochondrial PLIN5 enrichment following acute contraction to induce lipolysis and in acute contraction in both sedentary and endurance trained rats. Interestingly, the amount of mitochondrial PLIN5 enrichment is similar in both sedentary and endurance-trained rats similarly to the decreased intramuscular lipid content following electrical contraction. It is generally thought that following endurance training skeletal muscle relies more on endogenous lipids then exogenous fuel sources, which led us to believe that following endurance training mitochondrial PLIN5 enrichment would be greater in endurance trained rats. Contrary to our hypothesis, this was not found in our experiment. Future studies should work towards determining whether different intensities of acute lipolytic contraction following endurance training would elicit a change in mitochondrial PLIN5 enrichment. Also, whether PLIN5 is being recruited from the lipid droplet or the cytosolic environment to the mitochondria under lipolytic conditions is unknown. Using intracellular fractionation methods to isolate the nuclear, cytosolic and mitochondrial fraction of skeletal muscle will shed light on

whether PLIN5 is moving to the mitochondria, providing more support for the role for PLIN5 in regulating fatty acid transportation to the mitochondria.

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Appendix

A1. Histology (Preparation)

Equipment

- Cork (Labelled) for each muscle
- Embedding resin cryomatrix
- 2 methyl butane
- Liquid nitrogen
- Liquid nitrogen container
- Small metal bowl
- Forceps

Protocol: Muscle freezing

1. Prepare cork sections (1X1) large enough to fit muscle sample
2. With lipid nitrogen in its container, cool the 2-methyl butane in the metal bowl
   - Cool 2-methyl butane to -155°C
   - Listen for first and second sizzle of 2-methyl butane; the inner area of the metal bowl should become white and slushy
3. Place a blob of the embedding compound in the center of the cork and dip into the cooled 2-metyl butane to cool
   - submerge cork and embedding compound into 2-methyl butane until it begins to freeze along the perimeter
4. Orient muscle section in the center of the embedding compound so that it is completely submerged and have the sample standing so the muscle fibres are running perpendicular to the cork
5. Top off the top of the mound with the embedding compound so the muscle sample is completely covered
6. In one swift motion invert the set sample and drop into the cooled 2-methyl butane for 30-90 seconds
7. Store sample at -80°C (with labelled aluminum foil) until slicing.

Protocol: Muscle Slicing

1. Cool cryostat to -22°C
2. Equilibrate muscle samples in cryostat for approximately 20 minutes
3. At a moderate pace slice 3 transverse sections (serial if possible) per microscope slide with a thickness of 10µm
4. Allow slices to dry for 30 minutes before storage (-80°C)

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Shaw S. C., Jones A. D., and Wagenmakers J.M.A. (2007). Network distribution of mitochondria and lipid droplets in human muscle fibers. Histochem Cell Biol, 129:65-72

A2. Oil Red O Stain

Equipment

- Bowl and slide holder
- Microscope slides
- Microscope cover slips
- Kim wipes

Reagents

- Preservation in formaldehyde solution
  37% Stock solution
  Deionised water

- Oil Red O stock solution
  500 mg oil red O
  60% triethyl-phosphate
  *mix on heat

- Oil Red O working solution
  36% triethyl-phosphate
  12 ml oil red o stock solution
  8 ml deionised water
  Filter 3X with Whatman paper (#42)
  *mix on heat

- Phosphate-buffer saline (1X PBS)
  137mM Sodium chloride (NaCl)
  3mM Potassium chloride (KCl)
  8mM Disodium hydrogen phosphate (Na_2HPO_4)

Protocol

1. Thaw and air dry muscle samples for approximately 30 minutes
2. Preserve samples with 3.7% formaldehyde solution for 1 hour
3. During fixation incubation (1 hour period) prepare working solution
4. Wash formaldehyde off microscope slides under moving deionized water for 5 minutes and dab dry using kim wipes
5. Submerge slides in working solution (oil red O) for 30 minutes
6. Was working solution off microscope slides under moving deionized water for 5 minutes and dab dry using kim wipes
7. Once slides are dry pipette 10µl of 10% glycerol or antifade reagent (Prolong gold antifade; for fluorescence) and cover with cover slip
8. Let dry overnight in the dark

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A3. Western blotting

Muscle Homogenization

Equipment

- Glass potter and pestle
- Eppendorf
- Forceps
- Surgical scissors
- Liquid nitrogen
- Scale
- Lipid nitrogen container
- Metal bowl

Reagents

- Homogenization Buffer
  250mM sucrose
  100mM KCl
  5mM EDTA
  225mL dH20 (pH 6.8, top up to 250mL dH20

Protocol

1. Add protease inhibitor tablet (1, Roche) and one phosphatase inhibitor tablet (1, Roche) to 10mL of homogenization buffer and stir until dissolved
2. Pipette 200µL of prepared buffer into glass homogenizer
3. Tare scale with glass homogenizer and cut approximately 10-15mg of whole muscle into homogenizer and record weight
4. Adjust volume of prepared sample buffer to reflect 1mg of protein to 25µl of sample buffer
5. Homogenize muscle (approximately 30 plunges) until no visible muscle chunks
6. **keep on ice
7. Transfer to eppendorf (poke hole on top). Option to store at -80°C or continue to protein determination

Protein determination (Bradford Assay)

Equipment

- Eppendorf

- 96 well plates

Reagents

- Bovine serum albumin (BSA) (1mg/ml)
  **Can make a larger batch and store at -20°C

Protocol

1. Make a standard as described below. Pipette protein in triplicate into 96 well plate
2. Pipette 36µl of deionized water into an eppendorf with 4µl of whole homogenate/sample/mitochondria (10x dilution)
3. Pipette homogenate/sample/mitochondria in triplicate into the 96 well plate
4. Add 200µl of Bio-Rad protein assay dye reagent (diluted) into each well. Mix samples by re-suspension and let sit for 5 minutes
5. Read absorbance wavelength at 595nm
6. Determine standard curve (polynomial curve) to solve for the y-intercept (protein concentration)
7. Multiply y-intercept by 10 to determine protein concentrations for homogenates/samples/mitochondria
8. Use protein concentration from Bradford assay to prepare samples for Western blotting
   **see table below

Final Protein Concentration (mM)	Volume of BSA (µL)	Volume of dH2O (µL)
1	500 (leave as is)	500
0.5	500	500
0.25	500	500
0.125	500	500
0.05	100	900
0	0	1000

** Bradford Assay standard preparation

Total Volume (**TV**)	30-60 µl

Sample Volume (**SV**)	**SV**= **TV***(desired final [protein]/[protein])
Laemelli (sample) Buffer (**SB**)	**SB**=**TV**/[**SB**]
Water (deionized)	dH_20= **TV-SV-SB**

** Sample Preparation

Polyacrylamide Gel Electrophoresis (SDS PAGE)

Equipment

- Short and tall glass plates
- Glass plate holding apparatus
- Gel casting stand
- Gel cassettes
- Electrode holder
- Mini-tank
- Rectangular bowl
- Transfer holder
- Black and white sponge
- Filter paper
- Polyvinylidene fluoride (PVDF) membrane
- Plastic plates

Reagents

- 10x Running buffer
  250mM Tris Base
  1.92M glycine
  1% Sodium dodecyl sulfate (SDS)

- Laemelli (sample) buffer (3x)
  1.875mL Tris Base (1M solution)
  3.625mL dH2O
  0.6g SDS
  0.006g Bromo-blue
  3mL glycerol
  1.5mL beta-mercaptoethanol

- 10x Transfer buffer
  30.3g Tris Base
  14.4g Glycine

200mL methanol
790mL dH2O
10mL 10% SDS

- 10x TBS
  100mL of 10x TBS
  900mL dH2O
  4mL 25% Tween 20

Protocol: Electrophoresis

1. Wipe short and tall plates with methanol and kimwipe before use
2. Load glass plates into holding apparatus with short plate facing outwards
3. Prepare gels as described below
4. Make up ammonium persulfate (APS) solution (30mg into 300µL)
5. Add 100µL of APS and 10µL of tetramethylthylenediamine (TEMED) and mix WELL.
6. With a transfer pipette, pour RUNNING gel in between short and tall plate 8/10ths of the plate and top off with methanol and let harden.
7. Once running gel has hardened, remove methanol
8. Add 100µL of APS and 20µL of TEMED into stacking gel and mix WELL
9. With a transfer pipette, pour STACKING gel in between short and tall plate, topping it off
10. Insert combs to create wells in the gel and let harden
11. Once hardened Remove glass plates and place into gel cassettes (suction onto sides by moistening the perimeter of plates) with short plate facing inwards
12. Place gel cassettes into electrode assembly and secure tightly
13. Place complete apparatus into mini-tank and fill the inside of the gel cassettes with running buffer
14. Boil samples for 5 minutes and then place on ice for 5 minutes
15. Load standard (5-10µL) and samples into subsequent wells
16. Run electrophoresis/migration for 90 minutes at 120 Volts
    **look for small bubbles coming from wire at bottom of electrode assembly apparatus

Protocol: Transfer

1. Remove gel from tall and short glass plates and let equilibrate in transfer buffer
2. Soak PVDF membrane in methanol
3. Create transfer sandwich. Be sure to roll sandwich out before finishing
   – Black foam, white foam, 2 filter papers, membrane, 2 filter papers, white foam, and black foam

4. Close the sandwich holder and place in transfer apparatus inside of a tank
5. Fill the tank with the remaining transfer buffer so that the entire transfer is immersed in transfer buffer
6. Run transfer for 60 minutes at 100 volts

Protocol: Blocking and Antibody incubations

1. Remove membrane from sandwich holder and place in appropriate blocking solution for 1 hour on gel rocker
2. Decant blocking solution and add primary antibody diluted in solution overnight at 4°C with slight agitation
3. Decant primary antibody and wash membrane with TBST (approximately 20ml) 3x for 5 minutes
4. Add secondary antibody diluted appropriately with solution and let incubate at room temperature for 1 hour on gel rocker

Primary Antibody	**Molecular Weight (kDa)**	**Dilution**	**µL in 5ml**
PLIN2	50-52		5
PLIN3	47	1:1000	5
PLIN5	50	1:1000	5
B- Dystroglycan	43-50	1:500	10
SERCA 1	110	1:1000	5
SERCA 2	100	1:2000	5
COX IV	15	1:3000	1.67

Secondary Antibody	**Species**	**Dilution**	**µL in 5ml**
PLIN2	Anti-mouse	1:10000	1
PLIN3	Anti-rabbit	1:10000	1
PLIN5	Anti-guinea pig	1:10000	1
B-Dystroglycan	Anti-mouse	1:10000	1

SERCA 1	Anti-mouse	1:20000	1
SERCA 2	Anti-mouse	1:20000	1
COX IV	Anti-mouse	1:20000	1

Protocol: Chemiluminesence

1. Decant secondary antibody and wash membrane with TBST 3x for 5 minutes
2. Add 1ml of chemiluminescent horseradish peroxidise reagent (peroxide solution and luminal reagent) into plate and pipette over for membrane for 5 minutes
3. Transfer membrane onto overhead projector paper, marking the location of the weights of on the standard
4. Expose membrane

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A4. Subsarcolemmal mitochondria isolations

Reagents

- Solution 1
  100mM KCl
  40mM Tris HCl
  10mM Tris base
  5mM MgSO4
  5mM Na2EDTA
  1mM ATP
  pH 7.4 and bring to final volume with deionized water
  **TV .25L

- Solution 2
  100mM KCl
  40mM Tris HCl
  5mM Tris base
  mM Na2EDTA
  mM ATP
  1% BSA
  pH 7.4 and bring to final volume with deionized water
  ** TV .50L

- Solution 3
  100mM KCl
  40mM Tris HCl
  5mM Tris base
  mM Na2EDTA
  mM ATP
  pH 7.4 and bring to final volume with deionized water

- Sucrose and manitol solution
  220mM Sucrose
  70mM Mannitol
  10mM Tris HCl
  mM Na2EDTA
  pH 7.4 and bring to final volume with deionized water

Protocol

1. Using fresh muscle, dice with sharp scissors on an inverted petri dish on ice
2. Remove a small chunk (approximately 15mg) for citrate synthase assay
3. Prep small eppendorf with approximately 1mL of Solution 1
4. Weigh diced muscle and place in prepped eppendorf and continue to dice muscle
5. Transfer diced muscle into a glass homogenizer and top up with appropriate volume of solution 1 (1:20 dilution)
6. Homogenize until no visible chunks (approximately 20 plunges)
7. Transfer homogenate into glass centrifuge tubes
8. Spin sample for 10 min at 700g at 4°C
9. Retain supernatant; transfer into clear plastic centrifuge tubes (50ml). Discard pellet
10. Spin sample for 10 min at 14 000g at 4°C to isolate primarily subsarcolemmal mitochondria
11. Decant supernatant, retaining pellet.
12. Re-suspend pellet in 10x the volume of solution 2
13. Spin sample for 10 min at 7000g at 4°C
14. Decant supernatant and re-suspend sample in 10x the volume of solution 3
15. Decant supernatant and re-suspend sample in sucrose /mannitol solution

Mitochondrial Protein Purification

Reagents

- 5X SMEA buffer
  1.25M sucrose
  25mM MOPS
  5mM EDTA
  25mM NaN_3

Protocol

1. Isolate mitochondria as described above (A4.) until step 14
2. Pipette 1ml of 60% percoll® on top of mitochondrial pellet
3. Spin samples for at 20 000g with 0 deceleration for 1 hour at 4°C
4. Gently remove top layer of percoll®, discarding pipette and with new pipette remove middle layer of mitochondria and place into new eppendorf *
5. Spin sample at 20 000g with 0 deceleration for 5 hours at 4°C
6. Remove middle layer as described in (4)
7. Proceed to step 15 described in A4

*Option to freeze mitochondria at -80 before 5 hour spin

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A5. Co-Immuoprecipitation (Pierce protein immobilization kit)

Reagents

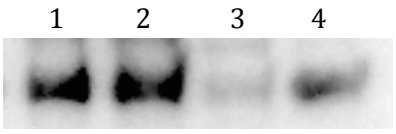
- Griffin lysis buffer
  150 mM NaCl
  50 mM TrisHCl
  1 mM EGTA

Protocol

1. Homogenize muscle samples as described in A3. homogenization with Griffin lysis buffer
2. Immobilize protein of interest by following instructions provided by Pierce Co-Immuoprecipitation (Co-IP) kit
3. Determine protein concentration once protein(s) of interest have been isolated as described in A3. Bradford assay and continue to SDS PAGE as described in A3.

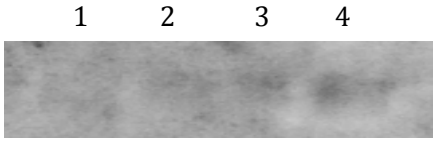
Representative Western blots

IP PLIN3 WB PLIN3



Lanes 1 and 2 loading IP PLIN3 samples, lane 3 was loaded with the supernatant from the PLIN3 sample and lane 4 was loaded with red gastrocnemius whole homogenate.

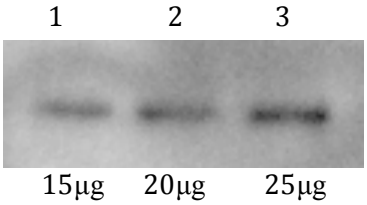
IP PLIN3 WB PLIN5



Membrane shown above (IP PLIN3 WB PLIN3) striped and re-probed with primary and secondary antibodies specific for PLIN5. As PLIN5 has proven to be a very sensitive protein

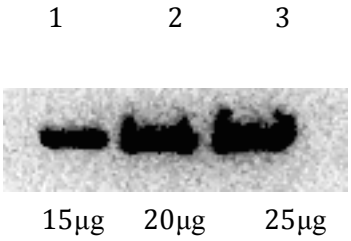
and can not undergo various freeze thaw cycles or constant agitation we were unable to obtain a clear representative Western blot for IP PLIN3 WB PLIN5.

IP PLIN5 WB PLIN5



Lanes 1, 2 and 3 are loaded with IP PLIN5 samples from red gastrocnemius muscles.

IP PLIN5 WB PLIN3



Membrane shown above (IP PLIN5 WB PLIN5) was striped and re-probed for primary and secondary antibodies specific for PLIN3.

Reference:

Elion E. A. (2006). Detection of protein-protein interactions by co-precipitation. Current Protocols in Neuroscience, 35, 5.251-25.10.

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